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Molecular Insights into the Uropathogenesis of Enterococcus faecalis and Escherichia coli in the Context of Urinary Catheterization

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Molecular Insights into the Uropathogenesis of *Enterococcus faecalis* and *Escherichia coli* in the Context of Urinary Catheterization

by

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DISSERTATION ABSTRACT

Molecular insights into the uropathogenesis of *Enterococcus faecalis* and *Escherichia coli* in the context of urinary catheterization

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Doctor of Philosophy in Biology and Biomedical Sciences
(Molecular Microbiology and Microbial Pathogenesis)

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Professor Scott J. Hultgren, Chairperson

Professor Michael G. Caparon, Co-Chairperson

Nosocomial urinary tract infections (UTIs) are a very common result of catheterization with 3 to 7% risk of developing catheter-associated UTIs (CAUTIs) each day catheterized. As multi-drug resistance increases in uropathogens, it is imperative to better understand the effects of catheterization on the urinary tract and pathogenesis. Further, microbial colonization and biofilm production on the surface of urinary catheters are a common component of CAUTIs. Thus, this dissertation focuses on understanding the contribution of known biofilm determinants to urovirulence of *Enterococcus faecalis* (a common nosocomial uropathogen) and uropathogenic *Escherichia coli* (UPEC) (the most common causative agent of UTI), and the effects of catheterization on bladder physiology using *in vitro* and optimized *in vivo* models. *In vitro* studies reported here demonstrated that *E. faecalis* produces DNA-dependent biofilms, which require both SrtA and Atn for efficient attachment as well as extracellular DNA from autolytic processes during the accumulative phase for maturation and architectural stability of the

biofilm under both static and hydronamic conditions. Further, *in vivo* studies optimizing and using a rodent model of foreign body-associated UTI to mimic conditions of indwelling urinary catheters in humans underscored the importance of biofilm formation, although *in vivo* required only a subset of the identified *in vitro* biofilm-promoting factors such as sortases, for the establishment of persistent enterococcal UTIs despite the acute inflammatory response ensuing from urinary implantation. This response was characterized in these studies and shown to involve bladder wall edema, partial disruption of the epithelial layer, vascular permeability, production of pro-inflammatory cytokines, and recruitment of myeloid cells, particularly neutrophils. During infection of implanted murine bladders, it was shown that type 1 pili mediate UPEC adherence and invasion, similar to what was observed in non-catheterized bladders. Studies in this murine model further demonstrated that microbial reservoirs established during previous UPEC infection could serve as a nidus for urinary catheter colonization upon subsequent implantation. Specific targeting of type 1 pili with small molecule inhibitors of the pilus tip adhesin, FimH, in combination with trimethoprim/sulfamethoxazole prevented UPEC CAUTI. This finding establishes a proof-of-principle for the development of novel therapies to prevent and eventually treat UPEC CAUTI in the face of the rise of antibiotic resistant uropathogens.

Overall, the optimization and use of the murine model of foreign body-associated UTI represents a significant advance in the understanding of the pathophysiology of *E. faecalis* and UPEC uropathogenesis in CAUTIs and is a valuable tool for the identification of virulence factors, including enterococcal sortases and UPEC type 1 pili, involved in these infections. Further research is required to uncover other biofilm and

virulence determinants specifically required for UTIs as well as host factors that can serve as potential antimicrobial targets and biomarkers for the prevention, diagnosis, and treatment of enterococcal and UPEC CAUTIs.

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This thesis is dedicated to my mother, Celine A. Anelone, for all her sacrifices throughout the years and her unconditional love for me.

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INTRODUCTION

Urinary tract infections

With more than 11 million cases diagnosed annually and half a million hospitalizations[1], urinary tract infections (UTIs) are among the most frequent infectious diseases acquired in the community and healthcare settings[2,3]. The associated cost of these infections is estimated at $3.5 billion every year in the United States[1,4]. UTIs are an inflammatory bacterial infection of the urinary bladder and urethra (lower UTI or cystitis) or in the ureters or kidneys (upper UTI or pyelonephritis) that can be associated with symptoms including fever, abdominal and flank pain, and painful urination[5].

UTIs are classified as uncomplicated or complicated based on patient's medical history and the use of instrumentation. Uncomplicated UTI occurs mostly in healthy individuals with no abnormalities to the urinary tract. They typically affect young, sexually active and non-pregnant women with a lifetime risk of 60.4%[6]. A subset of these women will experience a recurrent episode within 6-12 months[7-10]. UTIs are also prevalent among infants and children and are associated with vesicoureteral reflux (VUR), severe renal dysfunction and scarring, and predisposition to recurrent infections[5,11]. On the other hand, complicated UTI is defined by the onset of local and systemic symptoms similar to those associated with pyelonephritis, including flank and back pain, fever, and malaise, occurring in both men and women of all ages that have functional or anatomic abnormalities in their urinary tract, the immunocompromised, those suffering from other medical conditions such as nephrolithiasis and fibrosis, or individuals undergoing long term urinary catheterization[5].

Catheter-associated urinary tract infections

Catheter-associated urinary tract infections (CAUTIs) are the most common complications resulting from the use of indwelling urinary catheters[12,13] and account for 40% of all nosocomial infections[14] with more than one million cases diagnosed annually in hospitals and nursing homes in the United States and an estimated cost of $600 million in medical expenditures every year[12,15,16]. CAUTIs are defined as symptomatic UTIs with urine culture of one or more bacterial species greater than 1,000 colony forming unit (CFU)/ml with neutrophils present or pyuria, otherwise 10^5CFU/ml[17]. The incidence of bacteriuria following urinary catheterization is extremely common and occurs at a rate of 3% to 10% daily[18,19] with a 3 to 7% risk each day of developing CAUTIs[20]. This high incidence of bacteriuria in catheterized patients complicates the diagnosis of CAUTIs because both asymptomatic and symptomatic patients will have bacteria present in their urine[21]. Yet both bacteriuria and pyuria, strong identifiers of uncomplicated UTIs, are not good correlates of CAUTI[22]. It is thus imperative to identify specific biomarkers of CAUTIs for better diagnosis and treatment.

Empirical antimicrobial therapy using oral antibiotics, including trimethoprim, cephalosporins, and fluoroquinolones, are often sufficient to alleviate symptoms of UTIs[23]. However, prevention and treatment are impeded by the ever-increasing rise in antibiotics resistance among uropathogenic strains. If left untreated, CAUTIs can lead to severe complications such as acute pyelonephritis, bacteremia, urosepsis, and in some cases, death[18,24]. The high incidence of CAUTIs coupled with their medical and economic challenges underscore the need for better understanding of CAUTI pathogenesis that can help in the development of more effective therapies.

Pathophysiology of CAUTIs

The pathophysiology of CAUTIs involves severe alterations of bladder homeostasis following urinary catheterization. Catheterization induces histological and immunological changes in the bladder leading to severe bladder wall edema, hematuria, urothelial hyperplasia, and immune cell infiltration even in the absence of bacterial colonization[25-30]. The insertion and presence of a urinary catheter also interferes with micturition causing urine pooling in the bladder, damages the urothelium preventing secretion of inhibitory factors such as Tamm Horsefall proteins and mucopolysaccharides, and alters urine characteristics such as osmolarity; all of which are innate defense mechanisms that prevent microbial growth in healthy bladders[31-34]. Thus, the disruption of the normal mechanical and antimicrobial defenses of the bladder following urinary catheterization[33,34] renders the bladder environment vulnerable to microbial adhesion, multiplication, and dissemination within the urinary tract[35,36].

Indwelling urinary catheters also provide an additional surface for microbial attachment and biofilm formation[37]. Biofilm on the surface of urinary catheters is a major component of the pathophysiology of CAUTIs and other chronic device-associated persistent infections such as cystic fibrosis and endocarditis[5,38-40]. These are bacterial communities encased within an extracellular matrix composed of carbohydrates, proteins, nucleic acids, and lipids[41,42]. The development of a biofilm is a complex multi-stage process depicted in Fig. 1[43]. It is initiated with a primary adhesion of the bacteria to a substratum, which is followed by the formation of microcolonies and production of an exopolysaccharide matrix, and finally culminates with the formation of a three dimensional multicellular mature structure[44].

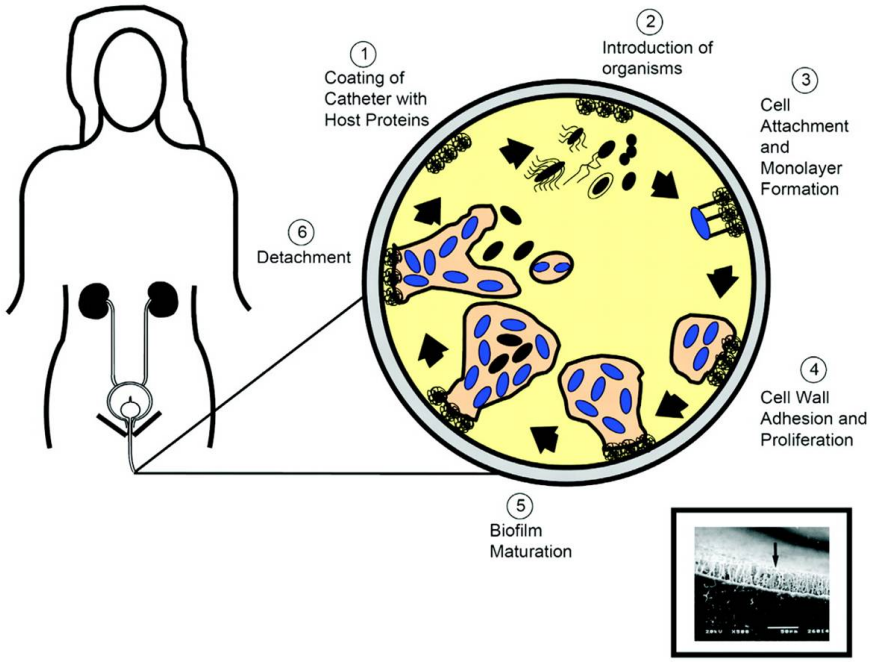

Figure 1: Proposed model of biofilm formation of the surface of urinary catheters

Following the insertion of the urinary catheter into the bladder, it is coated by host factors present in the urine. Bacteria introduced from the periurethral or extracellular milieu attach to the urinary catheters, multiply to produce microcolonies with secretion of an extracellular matrix. The microcolonies mature to form mature 3-dimensional structure, called biofilms. Eventually cells can detach or are shed for the biofilm to start the process again or disseminate to other tissues. Adapted from Reference[43].

Once formed, biofilms constitute an ideal environment for exchange of genetic material, such as genes encoding virulence factors and antibiotic resistance determinants among bacteria within the community[45,46]. Additionally, biofilm matrices allow for the flow of water and nutrients as well as ions and various small molecules to bacteria within the community[42,47] and provide a protective shield against antibiotics, antimicrobial

substances, and host immune defenses such as phagocytosis[48-54], leading to chronic or recurrent infections that are difficult to treat.

Etiologic agents of UTIs

Uropathogenic *Escherichia coli* (UPEC) is the major etiological agent of all UTIs, responsible for 80% of community-acquired UTIs and over 50% of nosocomial UTIs[55]. Emerging uropathogenic strains including *Pseudomonas*, *Klebsiella*, *Staphylococci*, and *Enterococci* are responsible for the remainder of UTIs, especially in nosocomial settings. *Enterococcus faecalis* and *Enterococcus faecium*, responsible for 15 to 30% of CAUTIs, are now the third leading cause of hospital-acquired UTIs[18,56]. UPEC and *Enterococci* are major etiological agents of CAUTIs[56] and are often isolated from mono and polymicrobial communities on the surface of urinary catheters with other Gram-negative and Gram-positive bacteria, including *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Staphylococcus* species[57,58]. Given their prevalence, medical and economic burden, further research is warranted to understand the mechanisms underlying UPEC and enterococcal CAUTIs.

UPEC uropathogenesis

E. coli, a rod-shaped facultatively anaerobic Gram-negative bacterium, is a member of the microflora of the gastrointestinal tract in humans. However, subsets of these *Enterobacteriacae* have acquired virulence factors that enable the establishment of various infections in the human host, including sepsis, meningitis, diarrhea, and UTI. UPEC, the major cause of all UTIs, belong to the extraintestinal pathogenic *E. coli* (ExPEC) subsets. UPEC are equipped with an arsenal of virulence factors that ensure colonization of the urinary tract while evading the host immune defenses[43,59]. Of

particular interest are the surface adhesins and extracellular fibers, including curli, pili, Dr adhesins, antigen 43, cellulose and flagella, that mediate adherence to urinary catheters and epithelial cells in the urinary tract, promote biofilm formation and contribute to the establishment of cystitis and pyelonephritis[60,61]. The adhesive type 1 pili, encoded by *fim* and which display phase variable expression, are the most prevalent virulence factor present in 80 to 100% of UPEC strains [43].

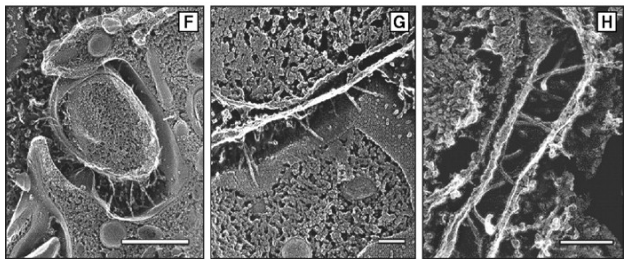

Figure 2: Type 1 pili are critical for UPEC adherence to uroepithelial cells.

Electron micrographs of type 1 pili mediating UPEC binding to uroepithelial cell surfaces prior to bacterial engulfment. Adapted from Reference[62].

Like many other bacterial fimbriae, type 1 pili shown in Fig.2 are assembled and translocated to the cell surface via the conserved chaperone usher pathway. Type 1 pili consist of the major subunit FimA that makes up the pilus shaft, the tip fibrillum composed of FimF and FimG subunits, and the tip adhesin FimH[63,64]. Type 1 pili mediate UPEC binding to uroepithelial cells[65] and are critical for UPEC infections[66]. The uropathogenic cascade of UPEC illustrated in Fig.3[67] has been extensively characterized using a murine model of cystitis[60,68,69]. It is a multistep process that is initiated with FimH of type 1 pili binding to glycoprotrein uroplakins and mannosylated residues on the surface of the superficial umbrella cells lining the bladder epithelium[70]. Following UPEC adhesion to urothelial cells, bacteria are internalized via zipper-like mechanisms involving local actin rearrangement and rhoGTPases[71,72]. UPEC replicate rapidly within bladder epithelial

cells to produce type 1 pili-dependent intracellular bacterial communities (IBCs) that possess biofilm-like properties[73,74], including positive periodic acid-Schiff (PAS) staining, differential gene expression, and morphological differentiation[67,73,75]. Upon IBC maturation, rod-shaped and filamentous bacteria then flux out from the infected cells and reinvade neighboring cells and start the process *de novo*[73].

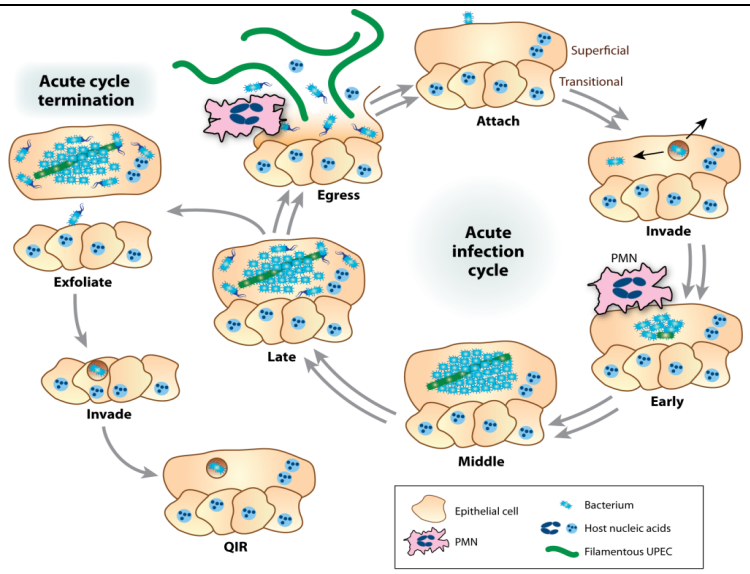

Figure 3: Schematic of the acute phase of UPEC uropathogenic cascade.

Following attachment and invasion of the superficial umbrella cells of the bladder, UPEC replicate, undergo morphological changes and develop intracellular bacterial community (IBCs) composed of coccoid and filamentous bacteria. Upon maturation, bacteria emerge from the infected cells and can reinvade neighboring cells to start the cycle over or establish quiescent intracellular reservoirs (QIRs) that can persist for a long period of time in the bladder. Reproduced with permission from Reference[67].

This acute phase of UPEC infection can eventually lead to the development of chronic cystitis, pyelonephritis, and the formation of quiescent intracellular reservoirs (QIRs) with absence of bacteriuria, which is postulated to be a source of persistent and recurrent

infections[76,77]. IBCs are also present in exfoliated uroethelial cells in urine from UTI patients, indicating that this pathway occurs during human infections[78]. The occurrence of this uropathogenic cascade following urinary catheterization as well as the role of type 1 pili and UPEC biofilm in CAUTI have yet to be characterized.

The detailed understanding of the molecular mechanisms underlying UPEC biofilm formation and uropathogenesis has led to the development of small molecule inhibitors targeting the assembly (pilicides and curlicides) and function (mannosides) of UPEC adhesive structures[79-82]. Pilicides and curlicides are dihydro thiazolo ring-fused 2-pyridone derivatives that block protein-protein interactions required for the assembly of type 1 pili and of curli amyloid fibers[80,83]. In contrast, mannosides are specific type 1 pilus adhesion, FimH, antagonists rationally designed to interfere with FimH interaction with mannosylated residues present on the surface of the uroepithelium[80,81,84-86]. These small molecules display anti-biofilm activity against UPEC *in vitro* and significantly attenuate virulence in the urinary tract[80,83]. Furthermore, mannosides can be used in combination with existing antibiotic therapy to prevent and treat UTI in mice[87]. Virulence-based therapeutic approaches may thus provide potential alternatives or supplement existing ones in the fight against UTIs, especially CAUTIs.

Enterococcal biofilm formation and urovirulence

Unlike UPEC, very little is known about enterococcal uropathogenesis. Enterococci are Gram-positive facultative anaerobic lactic acid-producing bacteria found as diplococci or in chains in the microbiota of the gastrointestinal tract of humans and animals[88]. They are non-spore forming bacteria that can withstand and grow in harsh environmental and physiological conditions, including high salt concentrations (6.5%

sodium chloride and 40% (w/v) bile salts), a wide range of pH (4.5-10.0) and temperatures (10-45ºC). These physiological properties and the production of antimicrobial peptides that are bactericidal to closely related species have led to the use of *Enterococcus* species for biotechnology and as probiotic agents in the food industry[88]. However, Enterococci, especially *E. faecalis* and *E. faecium,* are also important opportunistic pathogens causing 12% of nosocomial infections[89], including UTIs, endocarditis and bacteremia which has a high mortality rate up to 61%[90].

The ability of *E. faecalis* isolates to adhere to and develop biofilms on the surface of medical devices such as intravascular and urinary catheters shown in Fig. 2[91-94], coupled with the increasing resistance to virtually all antibiotics, including aminoglycosides and glycopeptides such as vancomycin[90,95-97], especially in biofilms[98,99]. Vancomycin-resistant enterococci (VRE) have been associated, since their identification in Europe in the 1980s[100,101], with several nosocomial infection outbreaks. In the United States, the Center of Disease Control and Prevention National Healthcare Safety Network reports that from January 2006 to October 2007, 4% of healthcare-associated infections result from vancomycin-resistant *E. faecium*[56]. This intrinsic and extrinsic resistance to antibiotics and the transfer of resistance genes to other species such Staphylococci[102] pose important clinical challenge in the fight against enterococcal infections. Several genetic factors and cellular processes, such as cell

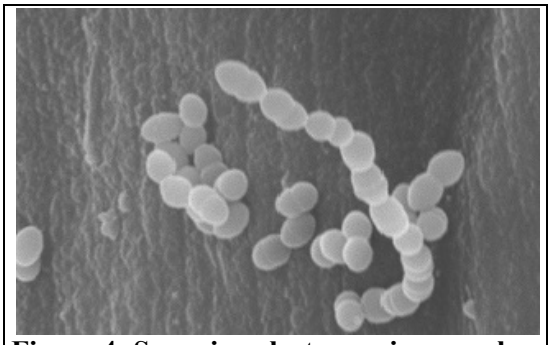

Figure 4: Scanning electron micrograph of *E. faecalis* strain OG1RF.

surface adhesins, proteases, quorum sensing, autolytic processes and extracellular DNA (eDNA), are implicated in biofilm development[91,103,104].

Although the mechanisms of biofilm formation in *E. faecalis* require further research, enterococcal biofilm determinants, including the enterococcal surface protein Esp[105], the pilus-associated sortase C (SrtC)[106], and the endocarditis and biofilm-associated pilus (Ebp)[107], contribute to enterococcal urovirulence. On the other hand, other well-characterized adhesins and *in vitro* biofilm determinants often associated with enterococcal UTI isolates like aggregation substance (AS) and the housekeeping sortase A (SrtA)[108,109] were reported to be dispensable for virulence in the urinary tract[108,109]. Since these conclusions were drawn from studies in murine models where persistent enterococcal infections cannot be established, it is imperative to re-examine the existing paradigm in an animal model that better mimics the transition of *E. faecalis* from a commensal to a virulent pathogen in the urinary tract.

Prospectus

The introduction of multidrug resistant Gram-positive and negative bacteria in the sterile environment of the bladder following urinary catheterization leads to the development of CAUTIs, major nosocomial infections recalcitrant to antibiotic treatment. Biofilm formation on the surface of indwelling urinary catheters is critical during CAUTI; however, limited research exists addressing the role of biofilm promoting factors in the establishment of CAUTIs, especially caused by UPEC and *Enterococcus* species. Studies addressing the hypothesis that known biofilm determinants of *E. faecalis* and UPEC are also involved in urovirulence following urinary catheterization and can serve as novel targets for virulence-based therapy in the prevention and treatment of these

infections are presented in the following chapters. The study described in this thesis provides molecular details of the temporal and spatial development of *E. faecalis* biofilms. Subsequent chapters describe the optimization and use of a murine model of CAUTI to assess the role of known biofilm factors to urovirulence of *E. faecalis* and the consequences of urinary catheterization on the uropathogenic cascade of UPEC.

CHAPTER ONE

ENTEROCOCCAL AUTOLYTIC PROCESSES AND SORTASES MEDIATE EXTRACELLULAR DNA-DEPENDENT BIOFILM FORMATION

Modified from: Guiton, P.S., *et al.* (2009) *Infect Immun*.

ABSTRACT

Biofilm production is a major attribute of *E. faecalis* clinical isolates. Although factors such as sortases, autolysis and extracellular DNA (eDNA) have been associated with *E. faecalis* biofilm production, the mechanisms underlying their contributions to this process requires further investigation. The present study defines important roles for the major *E. faecalis* autolysin (Atn), eDNA, and sortase A (SrtA) during the developmental stages of biofilm formation under static and hydrodynamic conditions. Deletion of *srtA* affects the attachment stage and results in deficiency in biofilm production. Autolysin-deficient mutants are delayed in biofilm development due to defects in primary adherence and DNA release, which is particularly important during the accumulative phase for maturation and architectural stability of biofilms. Confocal laser scanning and freeze-dry electron microscopy of biofilms grown under hydrodynamic conditions revealed that *E. faecalis* produces a DNaseI-sensitive fibrous network important for biofilm stability. This DNA-dependent fibrous network is absent in *atn*-deficient mtutant biofilms. Overall, this study establishes the stage-specific requirements of SrtA and Atn and demonstrates a role for the major enterococcal autolysin in the pathway leading to DNA release during biofilm development in this opportunistic organism.

INTRODUCTION

Bacterial biofilms can withstand harsh environmental stress conditions, including low nutrient availability, temperature fluctuations, antibiotic and antimicrobial treatments, and host immune responses[38,110,111]. Medical biofilms are associated with the establishment of chronic and persistent infections, including enterococcal CAUTIs[40,112,113]. The ability of *E. faecalis* to adhere to and develop biofilms [91] on medical devices, such as intravascular and urinary catheters, is thought to contribute to its pathogenesis.

Several putative virulence factors and cellular processes have been implicated in the development of biofilms in *E. faecalis*[91,92,114-121]; however, very little is known about their regul[91]ation and molecular contribution during this process. One of these factors, the quorum-sensing two-component transduction signaling system encoded by the *fsr* locus, an important virulence factor in the pathogenesis of *E. faecalis*, controls biofilm formation via positive regulation of the extracellular zinc metalloprotease GelE (gelatinase) and the serine protease SprE[115,117-120]. These proteases were recently shown to contribute to enterococcal biofilm formation via regulation of cellular autolysis and fratricidal DNA release[103,104]. In these studies, Thomas *et al.* also provided the first evidence for a critical role of extracellular DNA (eDNA) in *E. faecalis* biofilms[104]. eDNA is an important component of the extracellular matrix of bacterial biofilms, providing structural stability to the biofilm and protection against antimicrobials[122-128]. However, the temporal and spatial requirements of this macromolecule throughout the establishment and growth of *E. faecalis* biofilms is not well characterized.

Bacterial murein hydrolases, also referred to as autolysins, have been implicated in biofilm production[127,129]. They are important contributors to cell wall growth and regulation as well as several lytic processes[130,131]. Two autolysins of *Staphylococcus epidermidis*, AtlE and Aae, are adhesins that contribute to bacterial attachment to polymeric surfaces and biofilm formation via release of eDNA[127,132,133]. *E. faecalis* produces several autolysins, which were recently identified and characterized[134-137]. The major *E. faecalis* autolysin, Atn (also known as AtlA), is a predominant enterococcal antigen during human infections[138]. Atn is an *N*-acetylglucosaminidase[134] important for daughter cell separation[139]. Disruption of *atn* in *E. faecalis* results in increased chaining, defects in primary attachment, and decreased biofilm production[92,103,109,136,137,139]. Furthermore, inactivation of this autolysin partially protects *E. faecalis* against the lytic activities of the β-lactams penicillin (*in vitro)* and amoxicillin (both *in vitro* and *in vivo*), but does not attenuate virulence in mouse and rabbit models of peritonitis and endocarditis, respectively[139-141]. However, the contribution of Atn to DNA release during biofilm formation remains to be established. Recently, Thomas *et al.* provided evidence that inactivation of this autolysin results in a decrease in DNA release similar to that of gelatinase-deficient mutants[103]. Furthermore, they showed that GelE and SprE can differentially cleave Atn *in vitro*, and this processing may underlie the mechanism of cell death and DNA release in *E. faecalis* during biofilm formation.

Due to their role in cell wall regulation, autolysins may affect the localization of cell wall-anchored proteins, which can be important for adhesion during biofilm development. In most Gram-positive bacteria, membrane-anchored transpeptidase enzymes known as sortases are responsible for sorting and covalently anchoring proteins

bearing an LPXTG motif to the cell wall[142 143]. Thus far, only class A and class C sortases have been implicated in enterococcal biofilm formation and virulence[106,109]. Deletion of the gene encoding sortase C, *srtC* or *bps* (*b*iofilm and *p*ilus-associated *s*ortase), resulted in a significant reduction in biofilm production and attenuation of virulence in a mouse model of UTI, unlike deletion of *srtA*, which had minor effects under similar conditions[106]. However, given the ubiquitous nature of sortases and the limited knowledge of the activity and substrates of the sole SrtA characterized in *E. faecalis*, it is plausible that this enzyme may play an important role in *E. faecalis* physiology and/or pathogenesis under different conditions. For instance, SrtA was shown to anchor the plasmid-encoded protein Asc10, which is involved in the pheromone-induced aggregation of *E. faecalis*[144].

The present study uncovers the contributions of *E. faecalis* SrtA, Atn and eDNA to the major developmental stages from bacterial attachment to the establishment of mature biofilms under static and hydrodynamic conditions. The findings indicate that both SrtA and Atn contribute to efficient primary adherence to the abiotic surfaces while Atn and eDNA promote biofilm maturation and architectural stability during the accumulative stage. These findings argue for a role of Atn in the temporal regulation of DNA release. Under hydrodynamic shear forces, *E. faecalis* produces an Atn-dependent DNaseI-sensitive extracellular fibrous network. Collectively, this study underscores the importance of SrtA and Atn-mediated DNA release at different stages during the establishment of *E. faecalis* biofilms. As a critical component of the extracellular matrix of *E. faecalis* biofilms, eDNA may serve as a novel target for the dissolution of these structures.

RESULTS

E. faecalis biofilm development occurs in two major steps

In order to delineate the major stages of *E. faecalis* biofilm development, OG1RF was monitored for 72h to assess biofilm formation on polyvinyl chloride (PVC) coverslips in trypticase soy broth supplemented with 0.25% glucose (TSBG) at 37ºC under static conditions. Biofilms were visualized and quantified by CLSM and crystal violet-based quantification. CLSM images of 24h and 72h OG1RF biofilms stained with the DNA dyes SYTO9 and PI show a slow accumulation of the biofilm during the first 24h, which was followed by a rapid buildup and increase in density and thickness (Fig. 5A). Crystal violet-based quantification confirmed this observation and displayed a biphasic biofilm growth curve. This curve shows that the biomass accumulated at a slower rate between 0 and 24h followed by a significantly faster growth during the subsequent 48h with an approximately 6 fold increase in biomass from 24h to 72h (Fig. 5B).

eDNA is required for biofilm development

SYTO9 is a membrane permeable DNA dye whereas PI stains only eDNA and DNA of membrane compromised cells. The CLSM images show the presence of yellow patches throughout the biofilms, which are representative of SYTO9 and PI co-localization due to the presence of dead cells or eDNA in these structures (Fig. 5A and 5C). To determine whether eDNA contributes to biofilm development, OG1RF was grown statically in presence of varying DNaseI concentrations, ranging from 0 to 50µg/ml. DNaseI treatment severely impaired the ability of OG1RF to develop into mature biofilms at concentration as low as 0.5µg/ml (data not shown). OG1RF grown in

presence of 5µg/ml DNaseI produced 92.3% less biomass at 72h (p=0.0121 by Mann-Whitney U test) than untreated controls (Fig. 5B), which was in accordance with the reduction in volume observed determined by SYTO9 absorbance (Fig. 5D). The detrimental effects of DNaseI on biofilm development are not due to bactericidal or bacteriostatic activities as DNaseI did not inhibit OG1RF planktonic growth (data not shown). CLSM analysis further showed that OG1RF was able to attach to the substratum and formed microcolonies in presence of DNaseI; however, it failed to build the large compact 3D structures observed at 72h in untreated controls (Fig. 5C). In addition, approximately 10 to 15% of the cells present in the DNase I-treated samples stained with PI compared to the 30 to 50% in the untreated samples (Fig. 5D). The lower level of PI staining in biofilms treated with DNase I indicates a reduction in the number of dead cells and/or eDNA. Together, these findings suggest that eDNA in OG1RF biofilms contributes to the establishment of mature enterococcal biofilm structures.

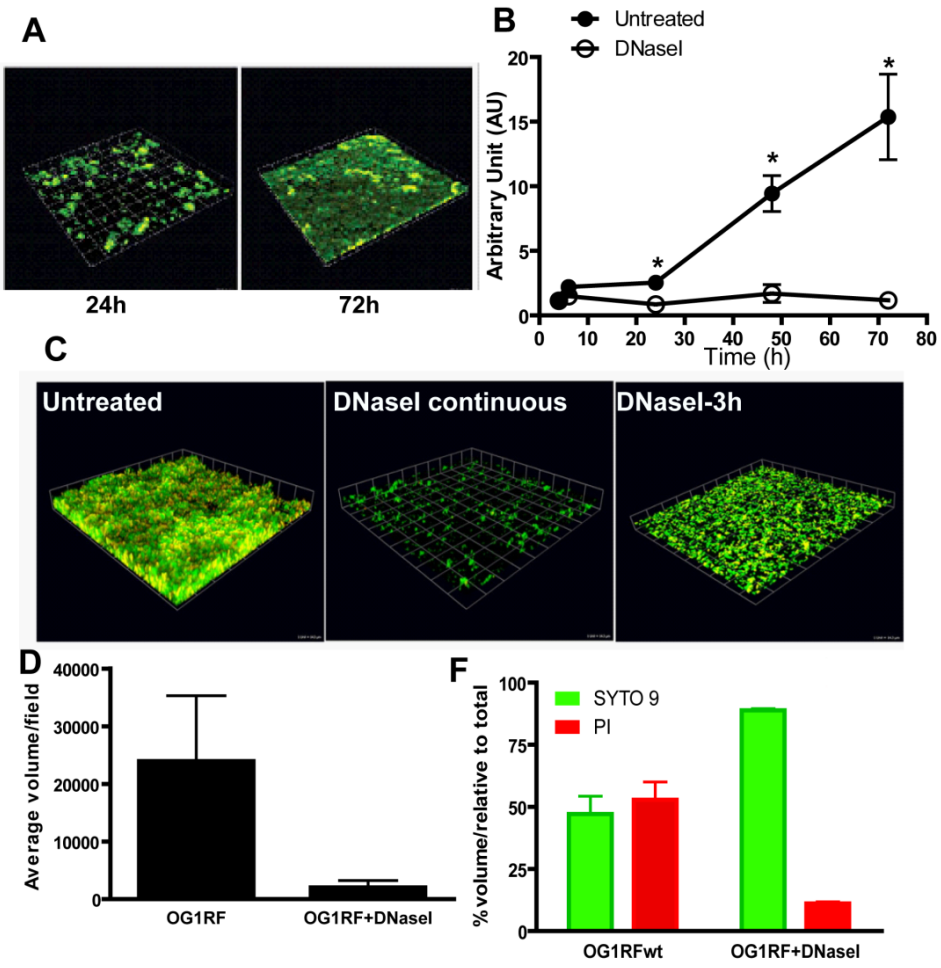

Figure 4: DNA is critical for *E. faecalis* biofilms under static conditions.

(A) CLSM images of OG1RF biofilms at 24h and 72h. Biofilms were stained with SYTO9 (green) and PI (red) and visualized with CLSM. 3D reconstructions of *z* stacks were generated with the Volocity software. **(B)** Crystal violet-based quantification of OG1RF biofilm biomass. Error bars = SEM of at least 3 independent experiments. *p<0.05, ***p<0.0005 by Mann-Whitney U test **(C)** CLSM images of 72h static OG1RF biofilms untreated or treated with 5 µg/ml of DNaseI continuously or for 3 h post formation. Scale, 1 unit on each side of the grid =14.3µm. **(D)** Quantification of biomass volume (μm^3) based on SYTO9 and PI absorbance using Volocity software. Graphs represents data obtained from a representative experiment with 3-4 randomly chosen fields. Error bars=SD

eDNA is required for maintenance of biofilm architectural integrity

To further characterize the function of eDNA in enterococcal biofilms, the effects of DNaseI on mature biofilms were investigated. Mature 72h static biofilms were treated with 5µg/ml of DNaseI for 1h, 2h, 3h, and 24h at 37°C followed by crystal violet staining or CLSM analysis. DNaseI treatment significantly reduced biofilm biomass by ~30% compared untreated controls 2h post treatment (p=0.0428) (Fig. 6A). Three hours treatment with DNaseI further reduced the biofilm biomass by 60% (p=0.008) while inactivated DNaseI (iDNase I) did not have any significant effects (p=0.7602; Fig. 6A). DNaseI inactivation was achieved following incubation at 75 to 100ºC and confirmed by its inability to degrade salmon sperm DNA (SSDNA) following 1h incubation at 37ºC (Fig. 6B). The biomass was further reduced to 40% of its original thickness (p=0.0003) 24h post treatment whereas iDNase I had no effect (p=0.5079; Fig. 6C). The disruption of mature biofilms following DNaseI treatment was also observed by CLSM (Fig. 5C and 6C). Disintegration of the biofilm structures occurred at 3h following treatment with 5µg/ml of DNaseI (Fig. 5C) compared to 1h with 50µg/ml DNaseI concentrations (Fig. 6C), suggesting that the effect of DNaseI on biofilms is dose and time dependent. Overall, these findings indicate that eDNA is an important structural component of *E. faecalis* biofilms and is necessary for maintaining their stability.

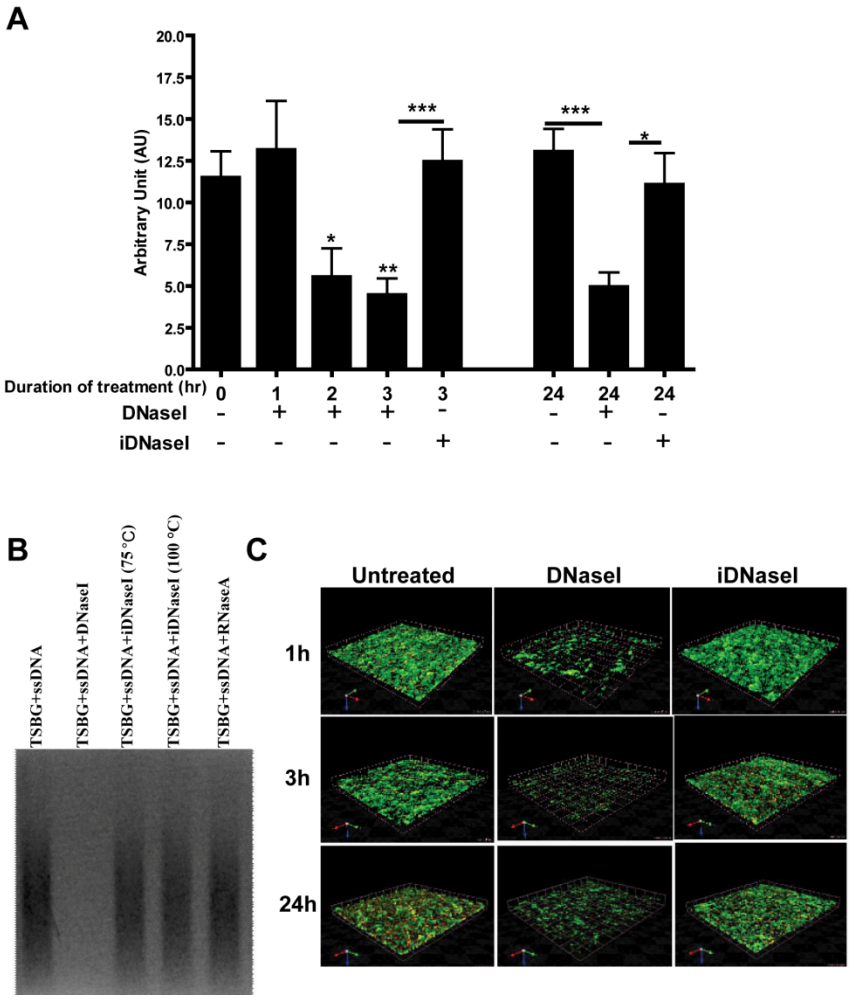

Figure 5: DNA is required for maintaining biofilm stability.

(A) Relative biomasses of 72-h OG1RF biofilms treated for 1 h, 2 h, 3 h, or 24 h with 5 µg/ml of DNase I or iDNase I at 37°C. Error bars = SEM from three different experiments. *$p < 0.05$ ** $p < 0.01$; *** $p < 0.0005$ by Mann-Whitney U test. **(B)** 0.8% agarose DNA gel showing the activities of DNaseI and heat inactivated DNaseI (iDNase I) at 75ºC and 100ºC for 20 min were assayed in TSBG at 37°C for 1h on 100µg/ml of salmon sperm DNA. **(C)** 72h old OG1RF biofilms stained with SYTO9 (green) and PI (red) were untreated or treated with 50 µg/ml of DNaseI or iDNase I for 1h, 3h, or 24h. Scale, 1 unit on each side of the grid =14.3µm.

***E. faecalis* forms a DNaseI-sensitive fibrous network under hydrodynamic conditions**

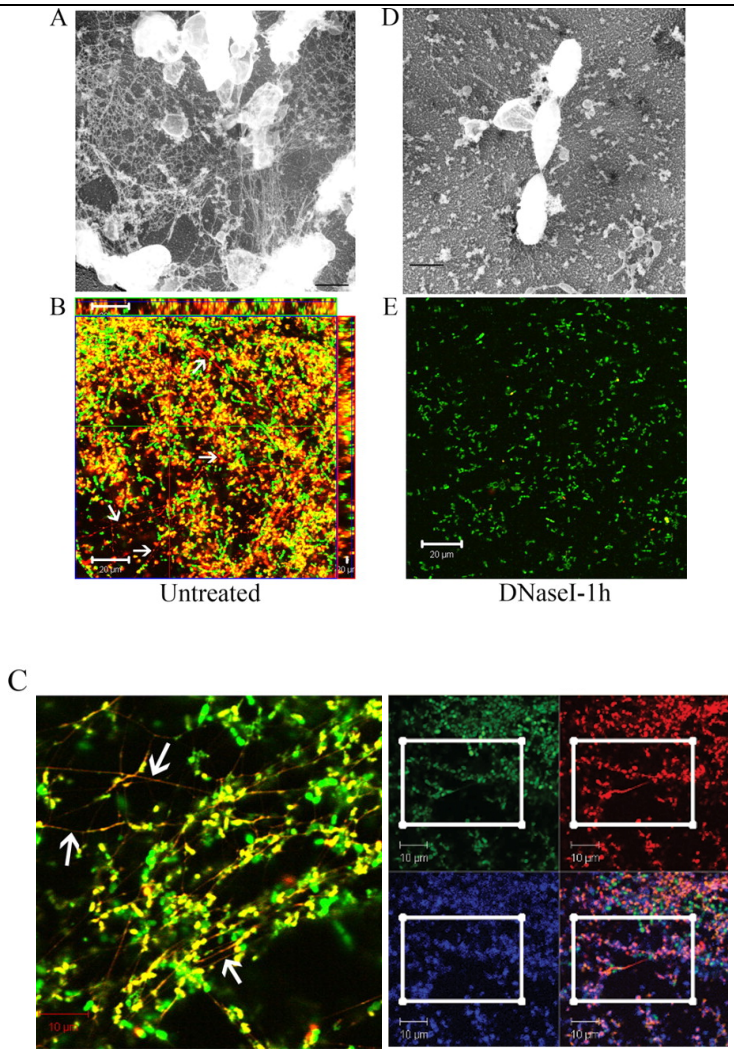

Figure 6: *E. faecalis* produces a DNaseI-sensitive extracellular fibrous network under hydrodynamic conditions induces the production.

The images are freeze-dry electron micrographs (Magnification, x20,000) **(A** and **D)** and CLSM images **(B, C,** and **E)** of 48h OG1RF biofilms untreated **(A, B,** and **C)** or treated for 1h with 5 µg/ml of DNaseI **(D** and **E)**. The arrows in **(B** and **C)** indicate SYTO9-PI stained fibers. Biofilm fibers (in white square) stain only with DNA dyes and not WGA. SYTO9 (green), PI (red), and WGA (blue). Scale bars = **(A** and **D)** 500nm, **(B and E)** 20µm, and **(C)** 10µm.

E. faecalis must withstand a variety of environmental conditions, such as urine flow in the bladder, for efficient colonization of medical devices. To investigate the effects of shear forces on *E. faecalis* biofilm development, OG1RF biofilms were cultivated in TSBG on PVC coverslips placed on an orbital shaker at 37ºC for the duration of the experiment. Freeze dry and CLSM micrographs of 48h dynamic biofilms show bacteria within the biofilm clustered via long fibrous structures under these conditions (Fig. 7A-C) that only stained with the DNA dyes and not with wheat germ agglutinin (WGA) that stains carbohydrates on cell surfaces (Fig. 7C). These extracellular fibers disappeared within 1h of DNaseI treatment (Fig. 8D-E), which correlated with disintegration of the biofilm. Additionally, immunogold transmission electron microscopy (TEM) analyses of OG1RF hydrodynamic biofilms labeled with mouse anti-dsDNA monoclonal antibody revealed foci inside and at the surface of cells as well as in the extracellular matrix surrounding the cells (Fig. 8A, top left panel), while examination of biofilms after 1 h of treatment with DNase I showed a reduced presence of eDNA (Fig. 8A, top right panel). Approximately 38% of the gold particles labeled the extracellular matrix compared to ~6% following DNase I treatment (Fig. 8B). Similar to the results for static conditions, OG1RF grown in the presence of 5 μg/ml of DNase I for 48 h failed to produce biofilms under hydrodynamic conditions and only 2% of the gold particles were found in the extracellular milieu under such conditions (data not shown). Treatment with proteinase K did not prevent OG1RF biofilm formation and did not affect the formation of the DNase I-sensitive extracellular fibers (data not shown). These results indicate that in the presence of hydrodynamic forces *E. faecalis* produces DNA-dependent biofilms with a distinct architecture from that of static biofilms. Additionally,

the data suggest that eDNA is a major component of the filamentous extracellular network in biofilms formed under hydrodynamic conditions that appears to provide cell-to-cell adhesion and stability in the biofilms.

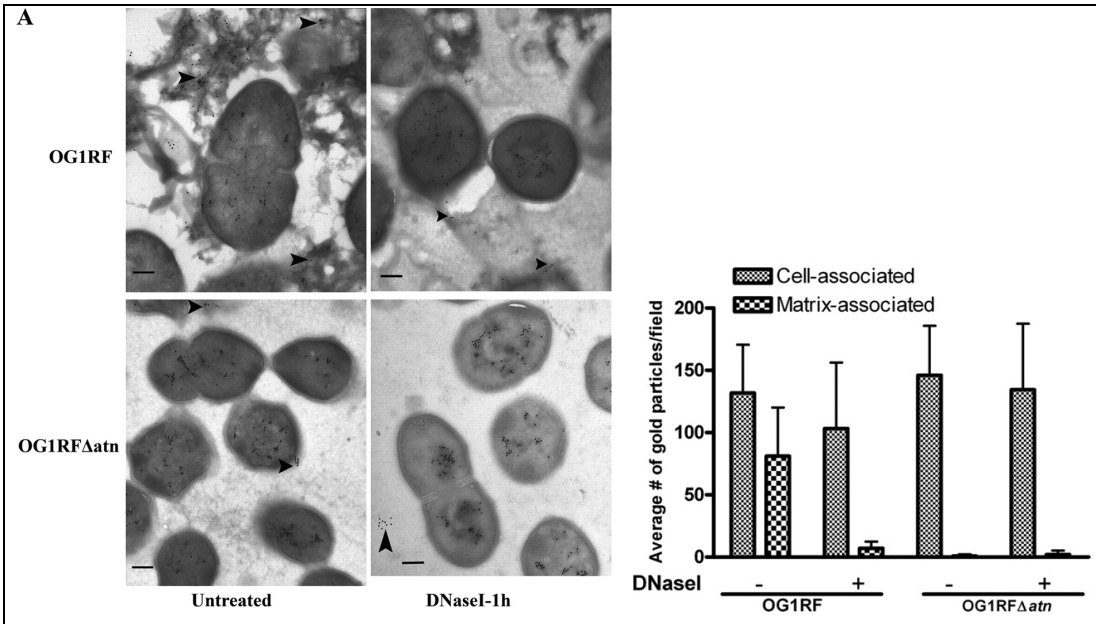

Figure 7: Atn contributes to DNA release during biofilm formation.

(A) Negative staining and immunoelectron microscopy images of 48h OG1RF and OG1RFΔ*atn* biofilms with or without 1h of DNase I treatment and labeled with mouse anti-dsDNA monoclonal antibody and secondary immunogold. The images are representative images of 10 random fields. Scale bars = 200 nm. Graph represents quantification of cell-associated or extracellular gold particles (indicated by arrowheads in panel **A**) from 10 independent fields for 48h dynamic biofilms at a magnification of x30,000.

Deletion of *atn* results in delayed biofilm formation

Freeze-dry electron micrographs revealed that lysed cells are constituents of enterococcal biofilms (Fig. 9A). This observation led to the hypothesis that Atn, the major autolysin of *E. faecalis,* facilitates DNA release and the formation of the DNase I-

sensitive extracellular fibrous network. To test this hypothesis, in-frame deletion of the autolysin gene (*atn*) was performed to generate an autolysin-deficient strain in OG1RF, OG1RFΔ*atn*. The ability of OG1RFΔ*atn* to form biofilms in the presence or absence of DNaseI was assessed. Deletion of *atn* did not affect OG1RF growth rate under normal culturing conditions (data not shown). As expected, based on the role of autolysins in cell lysis and cell division, OG1RFΔ*atn* had a reduced rate of cellular lysis as determined by a cellular autolysis assay under both static and hydrodynamic conditions (Fig. 9B) and formed longer chains than the parent strain (Fig. 9C). Compared to wild type OG1RF, OG1RFΔ*atn* was delayed in its ability to form biofilms under hydrodynamic conditions and produced approximately 40% less biomass by 48h as compared to that of the parental strain (Fig. 10A). Continuous DNase I treatment resulted in a ~40% reduction in the OG1RFΔ*atn* biomass accumulated by 48 h ($p = 0.038$) compared to an 87% reduction for the wild type ($p= 0.0004$; Fig. 10A). Similar levels of inhibition are observed following 1h of DNase I treatment of 48-h OG1RFΔ*atn* biofilms (Fig.10B-C). For OG1RFΔ*atn* biofilms, there was a 33% decrease in biomass ($p > 0.05$) following DNase I treatment compared to a 78% reduction for the wild-type structures ($p < 0.0001$) (Fig.10B). CLSM analysis of 48h dynamic OG1RFΔ*atn* biofilms revealed the absence of DNase I-sensitive extracellular fibers and very few PI-stained cells (Fig. 10C-D). Additionally, reduced amounts of eDNA were detected in the extracellular matrix of the mutant biofilms using cryoimmunoelectron microscopy with murine anti-dsDNA monoclonal antibody labeling (Fig. 8). Under static conditions, deletion of *atn* caused a similar reduction in biofilm biomass. The structure produced in the absence of *atn* was significantly more resistant to DNaseI treatment compared to wild type biofilms (Fig.

10A-C) and the cells within the static biofilms were loosely packed (Fig. 10B). Complementation studies could not be performed with OG1RFΔ*atn* expressing *atn* from a vector, perhaps due to the potential cytotoxic effects of Atn in the recipients, as previously suggested[137]. Nonetheless, the above findings indicate that Atn and cellular lysis play a role in DNA release during enterococcal biofilm formation. However, the ability of OG1RFΔ*atn* to form delayed and partially DNase I-sensitive biofilm suggest the existence of Atn-independent mechanisms of DNA release.

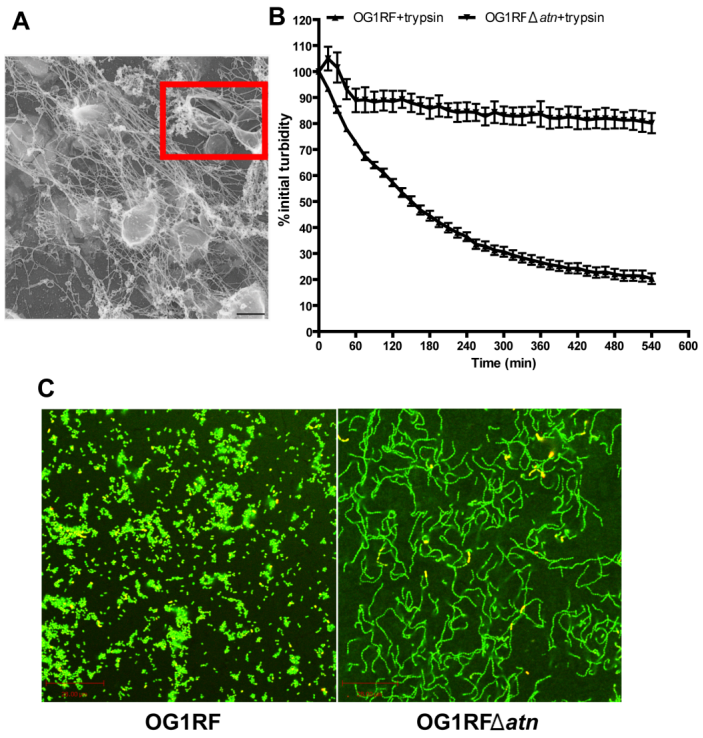

Figure 8: Effects of *atn* deletion.

(A) Freeze-dry electron micrograph of a 48h dynamic OG1RF biofilm with extracellular fibrous network protruding from lysed cells (red square). Magnification, x30,000. Scale bar = 500 nm. **(B)** Cellular autolysis of exponential-phase OG1RF and OG1RFΔ*atn* in trypsin/sodium phosphate buffer. Error bars = SEM from at least 3 independent experiments **(C)** OG1RF and OG1RFΔ*atn* cells from 24h TSBG liquid cultures stained with SYTO9 and PI. Scale bar= 10 µm.

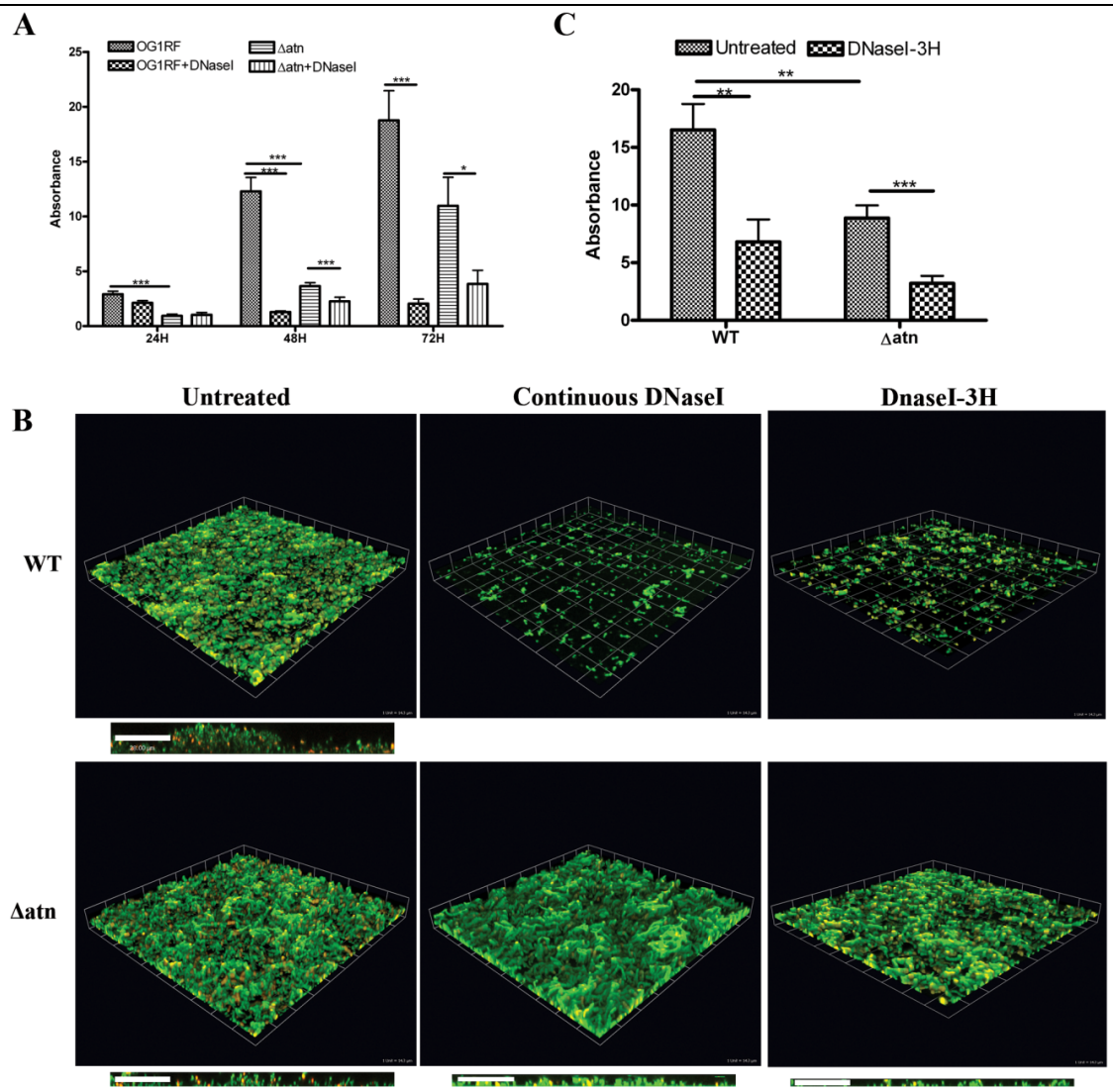

Figure 9: Characteristics of OG1RF and OG1RFΔatn grown in static conditions. (A) Crystal violet-based quantification of OG1RF (WT) and OG1RFΔ*atn (*Δ*atn*) biofilms with or without DNaseI treatment at indicated times. (B) CLSM images of 72h WT and Δ*atn* biofilms untreated, treated continuously or post formation with 5 µg/ml of DNaseI. The cross-section images reveal the thickness of the biofilms. Scale bar = 28 µm. (C) Crystal violet –based quantification of 72h WT and Δ*atn* biofilms treated with or without DNaseI for 3h *p<0.05, **p<0.005, ***p<0.0005 by Mann-Whitney U Test.

Atn contributes to DNA-independent attachment of *E. faecalis* to plastic surfaces

To assess the role of eDNA in initial attachment of *E. faecalis* to plastic cover slips, a primary attachment assay was performed in presence or absence of 5µg/ml of DNaseI under static and hydrodynamic conditions. Very few cells were adhered to the substratum after 2h in either condition (data not shown). By 4h and 6h, significantly fewer OG1RFΔ*atn* cells (40%) than wild-type strain cells were adhered to the surface ($p < 0.0001$; Fig. 11A), suggesting that Atn is required for this process. In contrast to *atn* deletion, DNaseI treatment did not affect the primary attachment at 4h for both wild-type strain OG1RF and OG1RFΔ*atn*. Similar levels of wild-type cells were attached to the surfaces regardless of the presence of DNaseI at 4 h ($p = 0.3527$) (Fig. 11A), indicating that eDNA is not critical for the initial attachment stage during biofilm development. Six hours of continuous DNase I treatment prevented microcolony formation (Fig. 11B), and this corresponded with a 33.3% reduction in the number of adherent cells. Albeit not statistically significant ($p = 0.4359$), this reduction suggests that DNaseI affects microcolony formation; microcolonies were readily visible at 6 h only in untreated samples (Fig. 11B). Furthermore, there was a >500% increase in PI staining from 4 h to 6 h compared to approximately 200% increase in the presence of DNase I (Fig. 11C), arguing that cell death and eDNA contribute to microcolony formation. Similar findings were obtained under hydrodynamic conditions (data not shown).

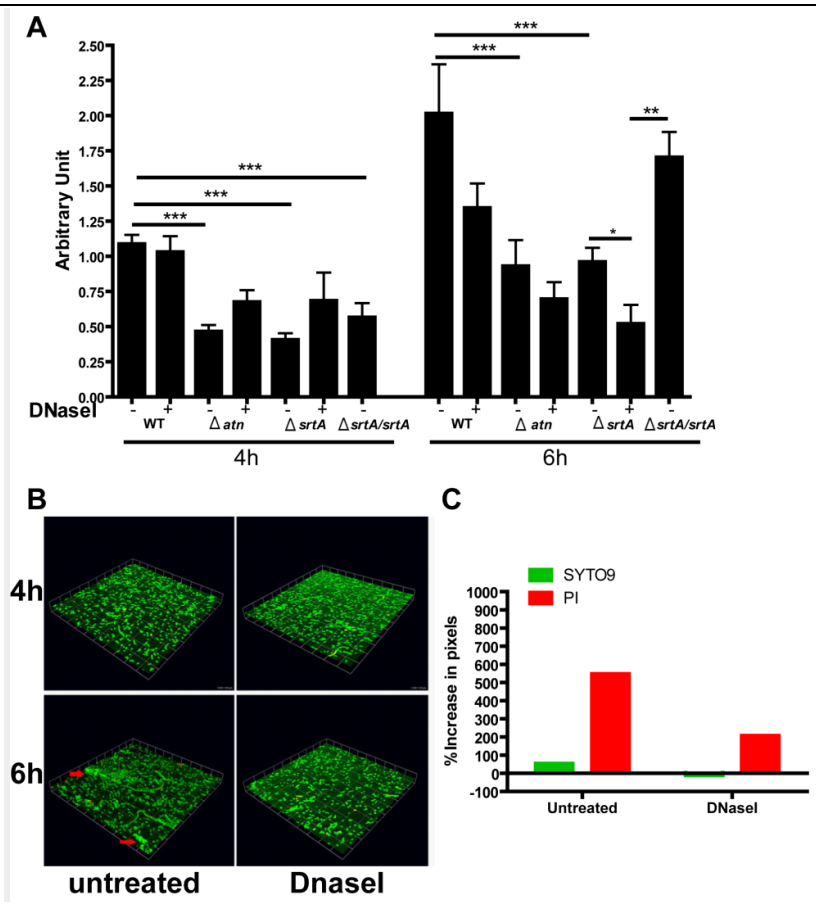

Figure 10: Primary attachment requires *srtA* and *atn*, but not eDNA.

(A) Primary attachment of OG1RF (WT), OG1RFΔ*atn* (Δ*atn*), OG1RFΔ*srtA* (Δ*srtA*), and OG1RFΔ*srtA/srtA* (Δ*srtA*/*srtA*) was determined by crystal violet staining following static incubation on PVC coverslips for 4 to 6 h at 37°C in TSBG with or without 5 µg/ml of DNase I. error bars =SEM for at least three independent experiments. *, $p < 0.05$**, $p < 0.01$; ***, $p <$ 0.0001 by the Mann-Whitney U test. **(B)** Representative CLSM images of 4h and 6h OG1RF (wt) adherent cells in the presence or absence of DNase I stained with SYTO9 (green) and PI (red). The arrows indicate microcolonies in 6h samples. One unit on each side of each grid equals 14.3 µm. **(C)** Quantification of the change in fluorescence of SYTO9 and PI relative to the total fluorescence from adhering cells from 4 h to 6 h post inoculation from 12 randomly chosen fields from two independent experiments using the VOLOCITY software.

Deletion of *srtA* results defect in primary adhesion

Given that autolysins are involved in cell wall growth and regulation, it is conceivable that deletion of *atn* causes mislocalization of adhesion factors critical for primary attachment and biofilm development. Sortases are ubiquitous enzymes with critical role in adhesion and virulence of Gram-positive bacteria. Given their role in cell wall protein sorting and anchoring, SrtA substrates on the cell surface may, hypothetically, contribute to initial stages of biofilm development. To investigate the role of SrtA in biofilm formation under static and hydrodynamic conditions, an in-frame deletion of *srtA* was generated in OG1RF, OG1RFΔ*srtA*. Deletion of *srtA* had no effect on cell viability and cell growth (data not shown). Similar to OG1RFΔ*atn*, OG1RFΔ*srtA* was also significantly deficient in primary attachment under static (p=0.0001; Fig. 11A) and shaking conditions (data not shown). Forty percent fewer OG1RFΔ*srtA* cells than parental strain cells adhered to the coverslips after 4h of incubation. The complemented strain OG1RFΔ*srtA/srtA* exhibited wild-type levels of adherence by 6h, showing significantly more adherence than OG1RFΔ*srtA* (p = 0.0022; Fig. 11A). It is not clear why the complementation effect was delayed and thus not observed at 4h; however, this may have been the result of plasmid loss in the complemented strain population, which allowed only a small subset to efficiently adhere to the substratum, leading to the observed delay. Furthermore, preliminary data from TEM analysis of OG1RF, OG1RFΔ*atn,* OG1RFΔ*srtA*, OG1RFΔ*srtA/srtA* grown statically in TSBG for 24h indicated that OG1RFΔ*srtA* produced fewer pili compared to wild type and the other strains (Fig. 12). Together, these findings suggest that SrtA and likely a SrtA substrate(s) play a role in promoting efficient initiation of biofilm development in *E. faecalis*.

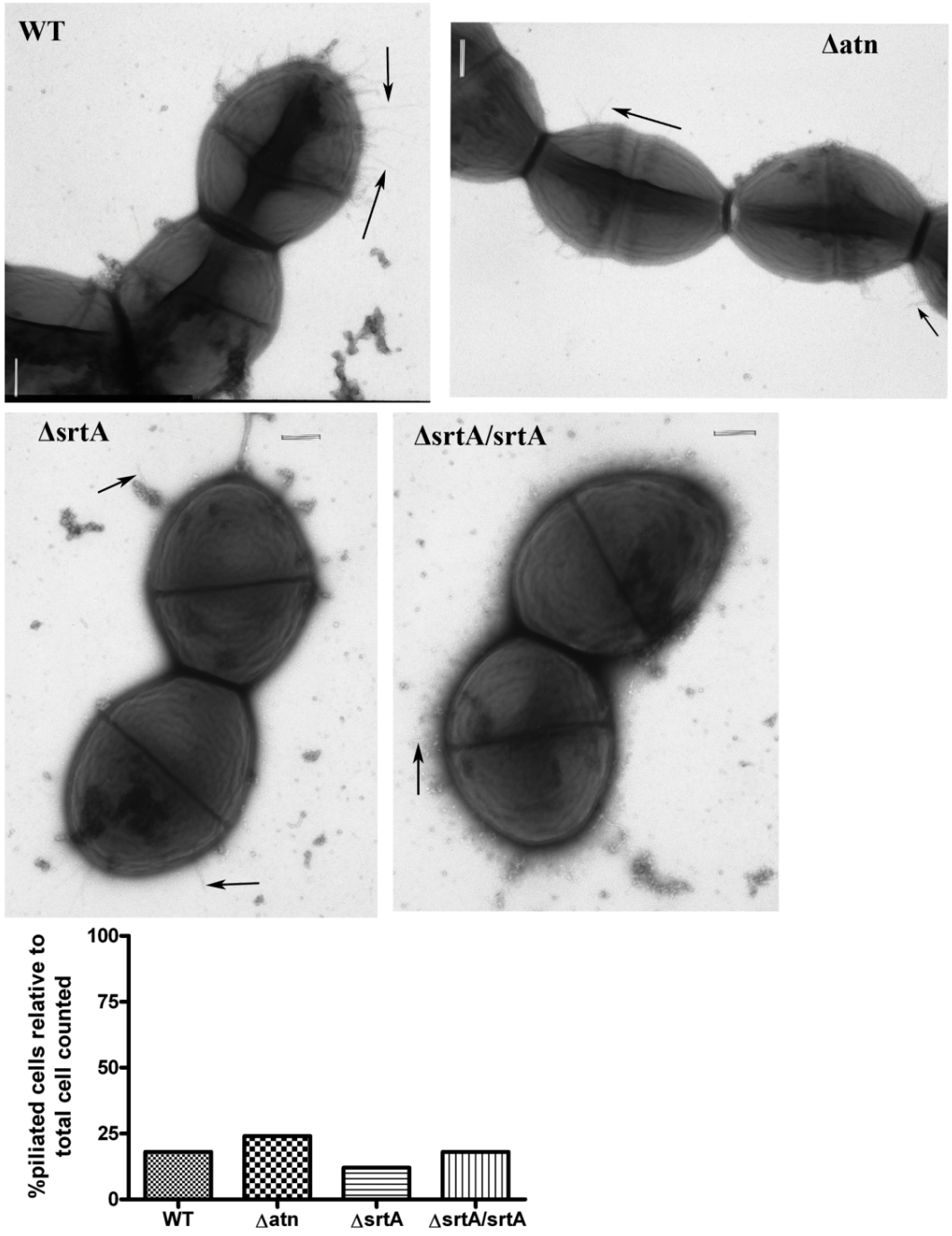

Figure 11: Piliation in OG1RF and isogenic mutants

Bacteria were grown statically in TSBG for 24h and prepared for TEM. Pili were counted on 500 cells per strain. Arrows indicate pili. Graph represents the percentage of piliation relative to number of cells counted. Magnification, Scale bar = Experiment was performed once.

srtA deletion results in defects in biofilm production

OG1RFΔ*srtA* was significantly defective in biofilm formation under static conditions; it produced ~60% less biomass than the wild type at 24 h ($p = 0.0142$) and even less (~40% reduction) biomass than the wild type at later time points ($p = 0.0244$; Fig. 13A). This defect in biofilm was complemented in OG1RFΔ*srtA/srtA* under static conditions (Fig. 13B-C). OG1RFΔ*srtA* and the complemented strain formed 3D biofilm structures that were sensitive 5µg/ml of DNaseI similar to wild type (data not shown). Additionally, the role of *srtA* in biofilm formation was assessed in presence of shear forces. In contrast to static conditions, the *srtA*-deficient mutant was severely defective under hydrodynamic shear forces (Fig. 13D). The impairment observed under these conditions was even more pronounced compared to static conditions. OG1RFΔ*srtA/srtA* exhibited a wild-type phenotype under these conditions (Fig. 13D), forming mature biofilms containing SYTO9-PI-stained extracellular fibers. These findings suggest that DNA release and extracellular fibrous network formation occur independent of SrtA.

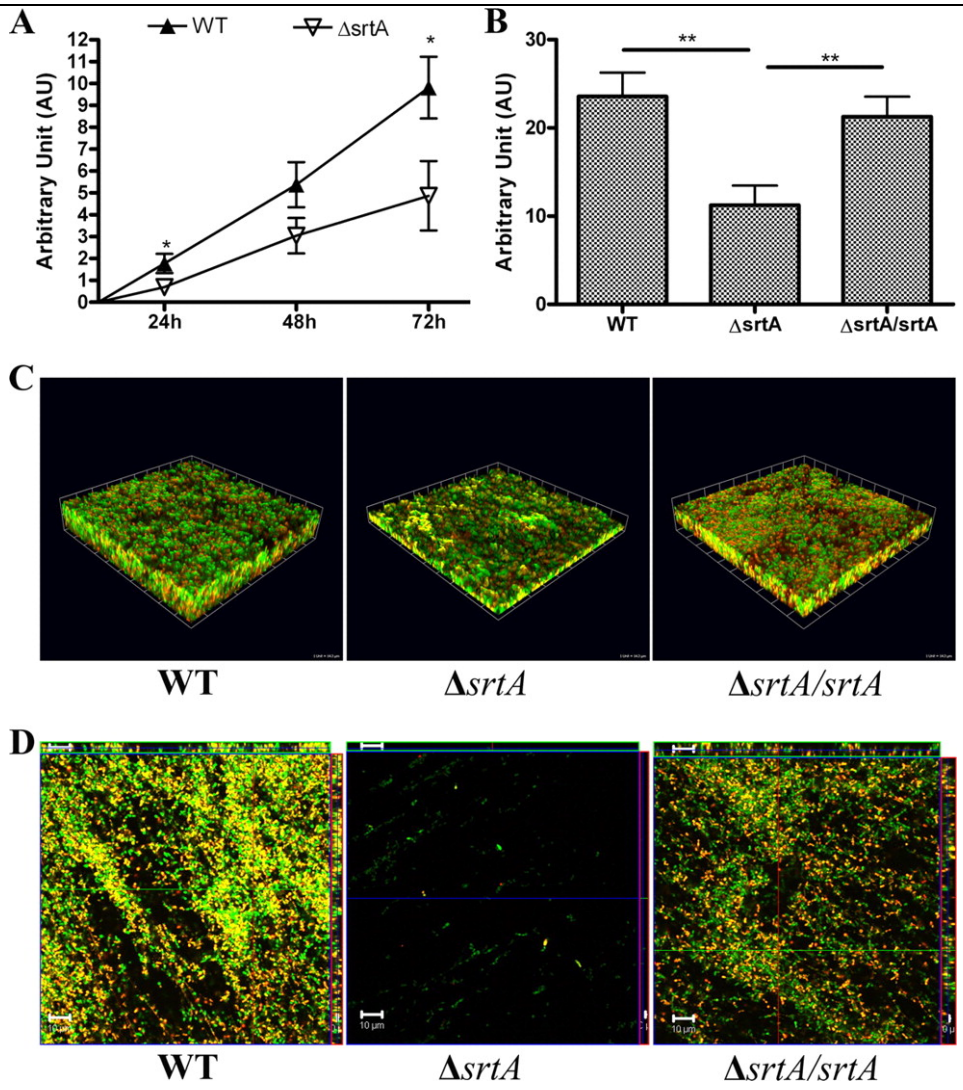

Figure 12: SrtA promotes high levels of DNA-dependent biofilm development.

(A) Crystal violet staining based quantification of OG1RF WT and OG1RFΔ*srtA* biofilm development and of **(B)** 72h OG1RF WT, OG1RFΔ*srtA*, and OG1RFΔ*srtA/srtA* static biofilms. Error bars = SEM of 3 different experiments. *p<0.05, **p<0.01 by Mann-Whitney's test. **(C-D)** 3D reconstruction of CLSM images of static 72h biofilms **(C)** and 48h dynamic biofilms **(D)** of OG1RF, OG1RFΔ*srtA* and OG1RFΔ*srtA/srtA* stained with SYTO9 (green) and PI (red). Scale, 1 unit on each side of the grid =14.3µm. White arrows indicate SYTO9-PI stained fibers. Scale bar=10µm.

DISCUSSION

Despite the identification of a myriad of genetic factors and cellular processes involved in *E. faecalis* biofilm formation[91,92,103,104,106,109,114,115,145-150], the molecular mechanisms underlying *E. faecalis* biofilm are yet to be defined. In this study, two major steps are delineated during *E. faecalis* biofilm formation: an initial attachment stage involving binding to an abiotic surface followed by an accumulative phase during which intercellular interactions to generate mature multicellular 3D biofilm structures. This report provides evidence of the temporal and spatial contribution of Atn-mediated autolytic processes and of the housekeeping sortase (SrtA) during biofilm formation.

The extracellular matrix of bacterial biofilms provides a safe haven for microbes within the community protecting them from various environmental stresses[42,47]. eDNA is a crucial structural component of the extracellular matrices of both Gram-negative and Gram-positive bacteria biofilms. This macromolecule mediates primary attachment, cell-to-cell adhesion, mature 3D biofilm architecture, and antimicrobial activities[122,125,128,151-153]. Thomas *et al.* showed that the vancomycin-resistant strain *E. faecalis* (V583) is unable to produce biofilms in presence of DNase I[104]. Similarly, *E faecalis* OG1RF strain, used in this study, is defective in biofilm formation following DNase I treatment. This enzyme can also disintegrate mature OG1RF biofilms, implying that eDNA is an important component of the extracellular matrix of *E. faecalis* biofilms and that this macromolecule is involved in cellular clustering during biomass accumulation. Although eDNA has similar functions under static and hydrodynamic conditions, its spatial contribution varies. Static biofilms are more compact with higher cell densities than biofilms formed in the presence of shear forces. Under hydrodynamic conditions, *E.*

faecalis produces a DNase I-sensitive fibrous network, reminiscent of a stable fibrous eDNA network of the aquatic Gram-negative bacterium strain F8[154,155]. Likewise, nontypeable *Haemophilus influenza* biofilms formed *in vivo* contain extracellular fibrous DNA networks[156] derived from neutrophil extracellular traps[157]. Notably, Kristich *et al.* also reported the presence of a filamentous extracellular network in enterococcal biofilms of both wild type and transposon-inactivated *atn* mutant strains grown statically on nitrocellulose membranes[109]. However, the composition of this structure was not characterized.

Previous reports have implicated Atn in primary attachment and biofilm production[92,109], but its contribution to this process, especially in DNA release, remains to be established. Atn is an important mediator of cell length and cell lysis during *E. faecalis* growth. Unlike autolysins described for other Gram-positive bacteria such as Aae and AtlE in *S. epidermidis*[127,133], Atn contributes to initial adhesion in a DNA-independent fashion. Inactivation of this autolysin leads to increased cellular chaining[139,158] that may reduce binding sites or increase cell surface charges causing lower cell deposition rate, thus explaining the adherence defects observed in *atn*-deficient strains. Repulsive interactions occurring between abiotic surfaces and bacteria as well as between adherent cells and incoming cells caused by surface charges were previously shown to influence the initial adhesion of bacteria to substrata[159,160]. Alternatively, defects in autolytic processes may alter the cell wall structure and localization of surface proteins required for attachment during biofilm formation, resulting in the deficient attachment and reduced biomass observed following *atn* deletion. In streptococcal species, biofilm production and bacterial colonization of abiotic surfaces are decreased

with disruption of *srtA* and genes encoding SrtA-dependent cell wall proteins[161,162]. The enterococcal housekeeping sortase (SrtA) is a biofilm-promoting factor. In contrast to previous reports for *E. faecalis*[106], deletion of *srtA,* in the present study, severely hampered adherence to plastic substrata and subsequent biofilm growth, especially under hydrodynamic conditions. These observations corroborate findings of Kristich *et al*. [109] in which transposon-mediated disruption of *srtA* results in approximately 30% decreased biofilm biomass compared to the parental strain using the microtiter biofilm assay. Together, these findings argue that SrtA plays a critical role in biofilm development in *E. faecalis*, especially in presence of shear forces. Sortase-dependent substrates have diverse functions ranging from adhesion and biofilm formation to immune evasion during pathogenesis[163,164]. LPXTG surface proteins, including aggregation substance (AS), the enterococcal surface protein (Esp), the *e*ndocarditis and *b*iofilm-associated *p*ilus (Ebp), a member of the *m*icrobial *s*urface *c*omponent *r*ecognizing *a*dhesive *m*atrix *m*olecules (MSCRAMMs) (ace), and the recently characterized EF3314, are the most studied proteins in *E. faecalis* and *E. faecium*. They mediate binding to host extracellular matrix proteins such as collagen and fibrinogen and promote enterococcal biofilm formation and virulence in animal models of enterococcal pathogenesis, including endocarditis and UTI[145,150,165-169]. Sortases and sortase substrates are thus attractive drug targets given that most of these cell wall proteins are involved in bacterial virulence. However, since *srtA*-deficient mutant biofilm are sensitive to DNase I treatment, it is unlikely that this gene plays a role in DNA release.

DNA release occurs from autolytic processes or competence induction in bacteria[121,126,151]. Freeze-dry electron micrographs in this study reveal extracellular fibers

protruding from lysed cells present in enterococcal biofilms. The findings reported here demonstrate that Atn is involved in DNA release during the accumulative phase of biofilm development in *E. faecalis*. This is in agreement with several reports highlighting the contribution of autolytic processes during biofilm formation[121,170,171]. Recently, the enterococcal proteases GelE and SprE, both under positive regulation of the *fsr* quorum sensing signaling, were shown to contribute to biofilm formation by regulating cellular autolysis[104]. GelE mediates DNA release during planktonic and biofilm modes of growth, in contrast to SprE, which acts as a negative regulator of autolysis and biofilm production. It is thus postulated that during biofilm formation fratricidal mechanisms underlie cell death and DNA release, which may result from the interactions between GelE and Atn[103,121]. However, certain *E. faecalis* laboratory and clinical strains, including OG1X further discussed in the second chapter of this dissertation, are able to produce biofilms in the absence of GelE[172], implying that *E. faecalis* may also employ GelE-independent DNA-dependent or independent mechanisms for biofilm formation.

Atn-mediated DNA release occurs at a specific time during the biofilm process. This finding implicates a temporal regulation of DNA release during biofilm formation. DNA release may occur in response to a particular cell density. It is possible that upon reaching critical mass following initial attachment, *E. faecalis* activates the *fsr*-quorum-sensing signaling pathway and the expression of autolytic factors, such as GelE and SprE. GelE cleavage of Atn, shown to occur *in vitro*[103], may then induce Atn-mediated cell death in a subpopulation[103] leading to DNA release and biofilm maturation and stabilization.

However, the ability of *atn*-deficient strains to produce DNA-dependent biofilms, albeit in a delayed fashion, is consistent with slower accumulation of eDNA from the actions of other autolytic factors in *E. faecalis*[134,173]. Mesnage *et al.* characterized two additional peptidoglycan hydrolases, AtlB and AtlC, in the JH2-2 strain of *E. faecalis*, which they showed to have compensatory functions in cell separation and autolysis in the absence of the major *N*-acetylglucosaminidase, AtlA or Atn[134]. It is likely that DNA release is the result of the synergistic activities of these autolytic enzymes during the establishment of *E. faecalis* biofilms. Further research is thus warranted to identify the regulatory pathway leading DNA release during biofilm formation.

Biofilm formation is a complex and highly regulated process involving a combination of genetic and environmental factors critical for specific developmental stages[174]. The present study uncovered a critical function for SrtA and provided strong evidence for the role of Atn in DNA release and biofilm production. Further research is required to identify SrtA-dependent proteins important for biofilm formation, in addition to those already characterized and involved in biofilm production, adherence to host cells and virulence in several models of enterococcal infections[166-169,175,176]. As an opportunistic pathogen, it is conceivable that *E. faecalis* employ several mechanisms to establish biofilms. eDNA is a critical component of the extracellular matrix of DNA-dependent biofilms produced by *E. faecalis* as well as *E. faecium* and *E. gallinarum* clinical isolates (data not shown). eDNA is crucial for maintaining the architectural integrity and the stability of enterococcal biofilm. The events mediating DNA release, localization, and interactions with other components of this protective structure require further investigation. Identifying the components and the regulatory

network of the extracellular matrix of enterococcal biofilms will be of great importance in the eradication of these pathogens.

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CHAPTER TWO

BIOFILM FORMATION IS CRITICAL FOR ENTEROCOCCAL VIRULENCE IN AN OPTIMIZED MURINE MODEL OF FOREIGN BODY-ASSOCIATED URINARY TRACT INFECTIONS

Modified from:
Guiton, P.S., *et al.* (2009) *Infect Immun*. and
Guiton, P.S., *et al*, manuscript in preparation

ABSTRACT

Catheter-associated urinary tract infections (CAUTIs) constitute the majority of nosocomial UTIs and pose significant clinical challenges. The insertion and presence of urinary catheters elicit major histological and immunological changes that render the bladder susceptible to microbial invasion, colonization, and dissemination. Enterococcal species are among the predominant causative agents of CAUTIs. However, very little is known about the pathophysiology of enterococci-mediated UTIs. This study optimizes a rodent model of foreign body-associated UTI in order to mimic conditions of indwelling urinary catheters in patients. Studies in this model uncover valuable details of the molecular constituents of foreign body-induced inflammatory responses and demonstrate their ineffectiveness against *E. faecalis* in catheterized animals. Enterococcal biofilm formation on the foreign body *in vivo* is essential for the establishment of persistent UTIs in the face of the massive inflammatory response ensuing from urinary implantation. Notably, only a subset of enterococcal *in vitro* biofilm-promoting factors, including sortases, is critical for biofilm formation and urovirulence. Overall, this murine model represents a significant advance in the understanding of CAUTIs and underscores the contribution of urinary catheterization to *E. faecalis* uropathogenesis. It is also a valuable

tool for the identification of biofilm and virulence determinants that can serve as potential antimicrobial targets for the treatment of enterococcal infections.

INTRODUCTION

Urinary catheterization is the primary cause of 80% of hospital-acquired UTIs[177]. The insertion and presence of indwelling urinary catheters disrupts the normal mechanical and host defenses of the urinary tract, allows extracellular microbes access into the sterile environment of the bladder by ascending through the catheter lumen or from the urethral meatus along the catheter, and provides an additional surface for biofilm formation and the establishment of persistent infections[30,32-36,178,179]. Even in the absence of microbial colonization, urinary catheterization is associated histological and immunological alterations in the bladder, including bladder wall edema, inflammatory cytokine production, immune cell infiltration, and mucosal lesions of the bladders and kidneys [26-30] which can lead to bladder cancers[180,181]. However, the molecular mechanisms underlying these responses require further research. The high incidence rate coupled with the medical and economic hurdles associated with catheter-associated urinary tract infections (CAUTIs) underscore the need for a better understanding of CAUTI pathogenesis.

Enterococci, infrequently associated with community-acquired UTIs, play a prominent role in the pathogenesis of CAUTIs[56]. *E. faecalis* is among the predominant pathogens isolated from polymicrobial communities on the surface of indwelling urinary catheters and biliary stents[182-186]. However, the mechanisms underlying *E. faecalis* uropathogenesis are poorly defined. Several rodent models have been developed to investigate *E. faecalis* pathogenesis in the urinary tract in single and mixed infections

with other Gram-negative pathogens[105,187-191]. These models involve the transurethral inoculation of low to high volumes of bacterial suspensions (50 to 200µl containing 10^7 to 10^8 CFU bacteria) into the bladders of healthy or streptozocin-induced diabetic animals[191]. The experimental conditions defined by these models are primarily useful for the study of *E. faecalis*-mediated pyelonephritis, but are inadequate for the investigation of persistent enterococcal CAUTIs as the bacteria are readily cleared from the bladder and fail to establish chronic cystitis[105,190,191]. Nonetheless, findings obtained from studies in these models have implicated several enterococcal factors in UTI pathogenesis, including the enterococcal surface protein Esp[105], the enterococcal *b*iofilm and *p*ilus-associated *s*ortase C (SrtC or *bps*)[106], and the *e*ndocarditis and *b*iofilm-associated *p*ilus (Ebp)[145]. However, well-characterized adhesins and biofilm determinants often associated with enterococcal UTI isolates[192,193] such as the aggregation substance (AS) and the housekeeping sortase (SrtA)[108,109], were reported to be dispensable for virulence in the urinary tract[106,108]. Since these conclusions were drawn from models where persistent infections cannot be established, it is imperative to re-examine the existing paradigm in an animal model that better mimics the transition of *E. faecalis* from a commensal to a virulent pathogen in the urinary tract.

This present study reports the optimization and use of a rodent model of foreign body-associated UTI developed in rats by Kurosaka *et al.*[29] and adapted in mice by Kadurugamuwa *et al.*[194] to investigate the pathophysiology of enterococcal CAUTIs. The report details unappreciated characteristics of the physiological changes in the bladder ensuing from urinary implantation. Findings from this murine model substantiate the allegations that biofilm formation is a major enterococcal virulence factor that is critical

for the establishment of persistent cystitis and pyelonephritis. Of the enterococcal *in vitro* biofilm determinants examined thus far, the enterococcal sortases and pili, as well as the enterococcal *e*nhanced *e*xpression of *p*heromone (Eep) metalloprotease and the transcription factor AhrC from the ArgR family, contribute to uropathogenesis in catheterized animals whereas the autolytic factors GelE and Atn are dispensable. This optimized murine model is thus well suited to identify host and enterococcal factors critical for pathogenesis in the urinary tract and will provide a better understanding of the mechanisms underlying the pathophysiology of CAUTIs.

RESULTS

Part I: Optimization and characterization of the murine model of foreign body-associated UTIs.

Urinary implantation elicits severe histological changes in the murine bladder akin to observations in human urinary catheterization. In order to study the pathophysiology of CAUTI associated with the presence of urinary catheters, 4-5mm long pieces of silicone tubing (implant) were transurethrally introduced into the bladders of seven to eight week old C57BL/6Ncr female mice as described in the Materials and Methods section and illustrated on Fig. 14. Silicone was chosen instead of polyethylene tubing[29] as it is the most common material used in All Silicone Foley urinary catheters in humans[195,196]. The recovery of silicone implants inside the murine bladders on the day of animals sacrifice was greater than 95% at 24h and approximately 75% seven days following implantation (data not shown). The data presented here represent animals with bladder implant at the time of sacrifice.

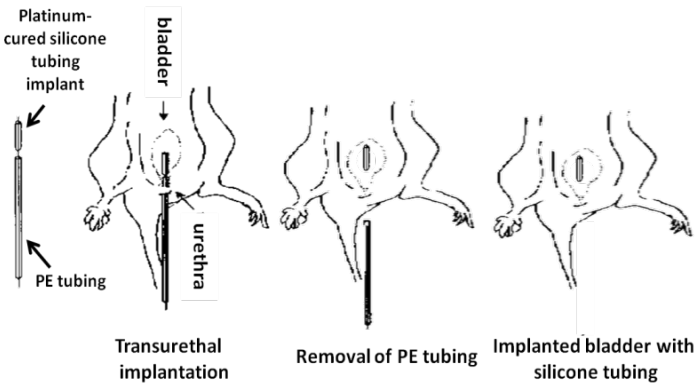

Figure 13: Schematic of transurethral urinary implantation.

Silicone implant is transurethrally introduced in the murine bladder by pushing a section of polyethylene tubing (PE) into the urethra with the help of tweezers. The silicone implant can remain in the bladder at least 7 days post implantation. Adapted from Reference [29].

Compared to non-implanted animals (Fig.15A and D), histological analyses of H/E stained bladder sections 24h post implantation reveal mucosal hyperplasia, submucosal edema with polymorphonuclear leukocyte (PMN) infiltration into the lamina propria and epithelium, and urothelial sloughing in implanted animals (Fig.15B and E). Damage to the uroepithelium in implanted bladders was also observed by immunofluorescence microscopy using anti-uroplakin III antibody, which stains the luminal surface of the terminally differentiated superficial umbrella cells of the bladder. Comparison of bladder sections at 24 h post implantation showed discontinuity of uroplakin staining of implanted bladders (Fig. 15H) versus continuous epithelial staining of non-implanted bladders (Fig. 15G), indicating the loss of superficial umbrella cells in the former. These data suggest that implantation with a foreign body elicits major

architectural changes in the bladder associated with activation of the host immune response.

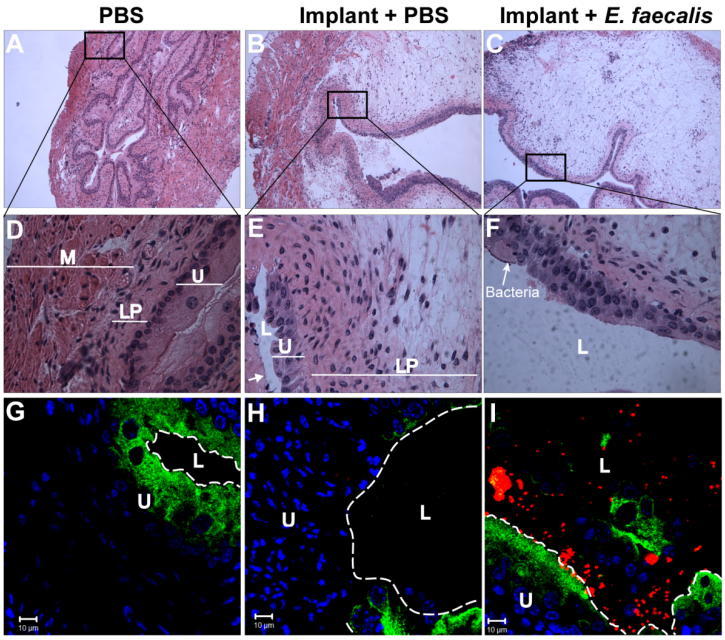

Figure 14: Implantation-mediated histological changes are associated with enterococcal colonization of the murine bladder.

(A-F) H/E staining of bladder sections from PBS-treated non-implanted **(A, D)**, PBS-treated implanted **(B, E)**, or OG1RF infected implanted **(C, F)** mice 24 hpi observed under light microscope at 40X (A-C) and 63X (D-F) magnifications. Arrow in **(E)** indicates damage to the uroepithelium. U=uroepithelium, LP=lamina propria, L=lumen, M=muscularis. **(G-I)** Representative CLSM images of the sections from the same animals as in **(A-F)** immunolabeled with goat anti-mouse uroplakin III (green), rabbit anti-Lancefield Group D antigen (red), and counterstained with TOPRO-3 nuclear dye (blue). Dashed line separates the lumen (L) from the uroepithelium surface (U). Scale bar =10µm.

Implant-mediated bladder edema and vascular permeability occurs independently of the neurogenic inflammatory pathway. The onset of bladder wall edema, classic

consequence of urinary catheterization in humans[30], was further assessed by bladder weight determination at 3, 6, 9, 12, 18, and 24h post implantation and compared to non-implanted animals. The murine bladder of implanted animals significantly increases in weight as early as 3h post implantation (p=0.0011 by the Mann Whitney U test) and reaches approximately 60mg by 24h (Fig.16A). Histological analysis of H/E stained bladder tissue sections from implanted animals at each time point depicts the gradual progression of bladder wall edema over time, corroborating the significant increase in bladder weight (Fig. 16B). The implant-induced edema can be attributed to plasma protein extravasation (PPE) in the tissue as assessed by Evans blue extravasation assay performed 6h post implantation (Fig. 16C). Approximately 60µg of Evans blue per gram of bladder tissue was extravasated and recovered from implanted bladders following intravenous injection in the murine tail vein, a significantly higher amount compared to 40µg/g from non-implanted controls (p=0.0348).

Bladder wall edema and vascular permeability are hallmarks of the neurogenic inflammatory pathway in the urinary tract in response to chemical irritants such as cyclophosphamide, capsaicin, and bacterial endotoxin[197,198]. The neurogenic inflammatory pathway is activated in response to the stimulation of sensory neurons innervating the bladder and the release of proinflammatory neuropeptides such as substance P, neurokinin A, and calcitonin gene-related peptide (CGRP)[197], which induces edema, neutrophil infiltration, the formation of reactive oxygen species, and contribute to pain[197,198]. This neurogenic inflammation can be inhibited by administration of specific small molecules inhibitors, including CP-99,994 and CP-96,345 which are specific neurokinin 1 receptor (NK1R) antagonists[199-202], aminoguanidine (AG) which is an

irreversible inhibitor of the inducible nitric oxide synthase (iNOS)[203-205], and alfuzosin which is an antagonist of alpha 1 adrenoreceptors present on the surface of sensory neurons[206]. To assess the contribution of the neurogenic inflammatory pathway in implant-induced edema, animals were treated with CP-99,994 or CP-96,345 (5-10mg/kg, i.p. or i.v.) and AG (200mg/kg, i.p.) administered respectively 30 min and 1h prior to implantation. A second dose of each compound was given 3h post implantation and PPE was assessed 6h post implantation. Phosphate buffered saline (PBS) or appropriate vehicle control was administered in the same manner as in the treatment group. No inhibitory effects were observed following treatments with neurogenic pathway inhibitory chemicals. PPE occurs at similar levels (~60ug/g) in compounds-treated implanted animals and vehicle-treated implanted animals (Fig.16C). Likewise, these treatments do not alter the increase in bladder weight post implantation (data not shown). Similar to the NK1R and iNOS inhibitors, preliminary evidence suggests that treatment with alfuzosin (60μg/kg, i.v.) does not prevent bladder weight increase nor inhibit vascular permeability in implanted bladders (data not shown). Additionally, no detectable levels of SP, the NK1R ligand that induces neurogenic inflammation, could be detected by mass spectrometry in implanted animals for 3h (data not shown). Immunofluorescence microscopy of implanted and non-implanted bladders at 6h and 24h stained with antibodies raised against SP and NK1R reveal no major changes in the expression or localization of these two mediators of neurogenic inflammation (data not shown). Together, these findings indicate that the NK1R-induced neurogenic inflammation is not a major contributor in the onset of bladder wall edema and vascular permeability.

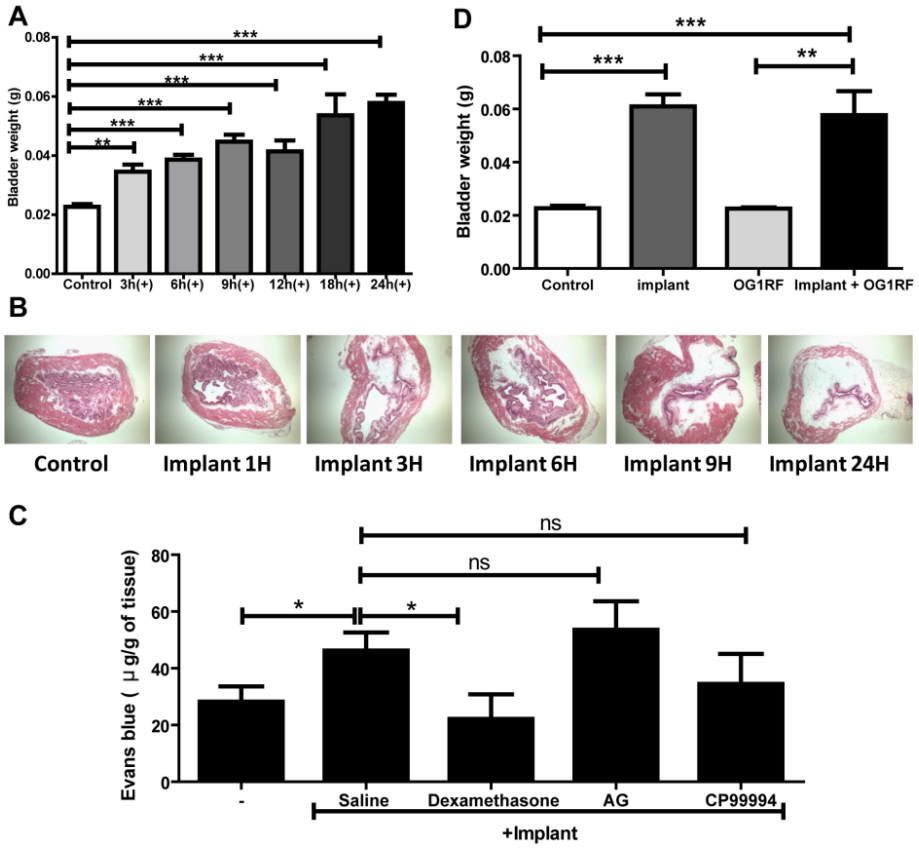

Figure 15: Urinary implantation induces edema and plasma protein extravasation.

(A) Bladder weights of non-implanted (control) and implanted (+)at the indicated time **(B)** H/E staining of bladder sections from non-implanted and implanted animals at indicated time points observed under light microscope at 10X magnification. **(C)** Plasma protein leakage in bladder tissue determined by Evans blue content at 6hpi in non-implanted animals treated with saline and implanted animals treated with saline, dexamethasone (10mg/kg, i.p.), aminoguanidine (AG, 2 doses of 200mg/kg i.p.), CP99994 (2 doses 5mg/kg, i.p.). **(D)** Bladder weights at 24hpi of non-implanted and implanted mice in presence or absence of *E. faecalis* OG1RF strain. Graphs represent the mean of each dataset from at least two independent experiments; Error bars=SEM; *$p<0.05$, ns: $p>0.05$ by the Mann Whitney U test.

Urinary implantation leads to the upregulation of specific inflammatory markers and the specific recruitment of myeloid-derived cells in the murine bladder. To identify the

soluble and cellular constituents of the bladder immunological responses to the silicone implant, multiplex bead arrays and flow cytometry assays were performed on bladder homogenates and suspensions in non-implanted and implanted animals at 24h post implantation. Of the 23 cytokines examined, only interleukin 6 (IL-6), G-CSF (Granulocyte-Colony Stimulating Factor), and KC (Keratinocyte-derived Cytokine) were consistently present in implanted bladder homogenates at least two-fold above the levels of non-implanted animals at 24 hpi (Fig. 17). Conversely, GM-CSF (Granulocyte Monocyte-Colony Stimulating Factor) and MIP-1α (Macrophage Inflammatory Protein 1 alpha) showed at least two fold decreased expression in implanted animals compared to non-implanted animals 24 hpi (Fig. 17). Flow cytometry experiments reveal that $CD11b^{+}Gr1^{+}$ myeloid cells account for approximately 15% of the live population in implanted animals compared to 1% in non-implanted controls ($p<0.0001$; Fig.18A). No difference was observed in lymphocyte numbers in presence or absence of implant at 24hpi (Fig. 18A). Specific markers were used to further specify the myeloid cells. This analysis reveals a 1-2 log increase in eosinophils ($SiglecF^{+}$), basophils ($FccR1^{+}$), and monocytes ($CD11b^{+}Ly6G^{-}Ly6C^{+}$) specifically in implanted animals (Fig.18B). Additionally, macrophages ($CD11b^{+}F4/80^{+}MHCII^{-}$ and $CD11b^{+}F4/80^{+}MHCII^{+}$) are present with approximately 1 and 1.5 log, respectively, over non-implanted controls (Fig. 18B). Neutrophils ($CD11b^{+}Gr1^{+}Ly6G^{hi}Ly6C^{lo}$) are the most abundant immune cells recruited in response to urinary implantation with approximately 3 log more cells relative non-implanted controls (Fig. 18B). Further studies using an antibody raised against the neutrophil-specific receptor NB1 (also known as CD177)[207] corroborates the above findings that PMNs are the major immune infiltrates in implanted murine bladders (data

not shown). There was no change in the number of dendritic cells ($CD11b^{+}CD11c^{+}MHCII^{+/-}$) or mast cells ($cKit^{+}$) present in implanted animals compared to non-implanted controls (data not shown). Together, these results indicate that the silicone implant elicits a specific inflammatory response in the bladder characterized by the up-regulation of pro-inflammatory cytokines and myeloid infiltrates.

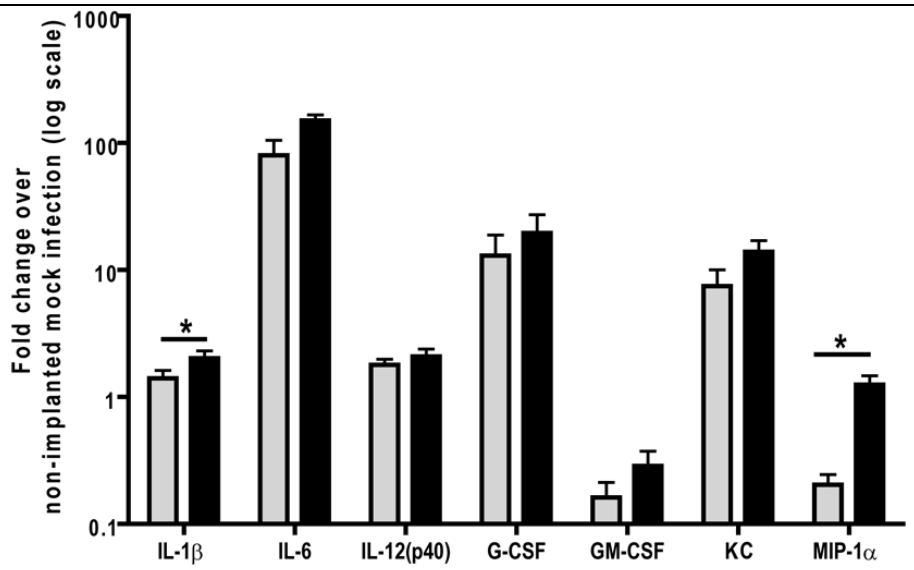

Figure 16: Cytokine profiles following urinary implantation with and without enterococcal challenge.

Graph represents whole bladder cytokine expression of implanted animals inoculated with PBS (gray bars) or OG1RF (black bars) at 24 hpi. Cytokines reported are expressed with at least two-fold difference relative to non-implanted controls. Data from a representative experiment of 4 independent experiments with n=5mice/condition/experiment. Error bars =SEM. *$p<0.05$, p-value by the Mann Whitney U test.

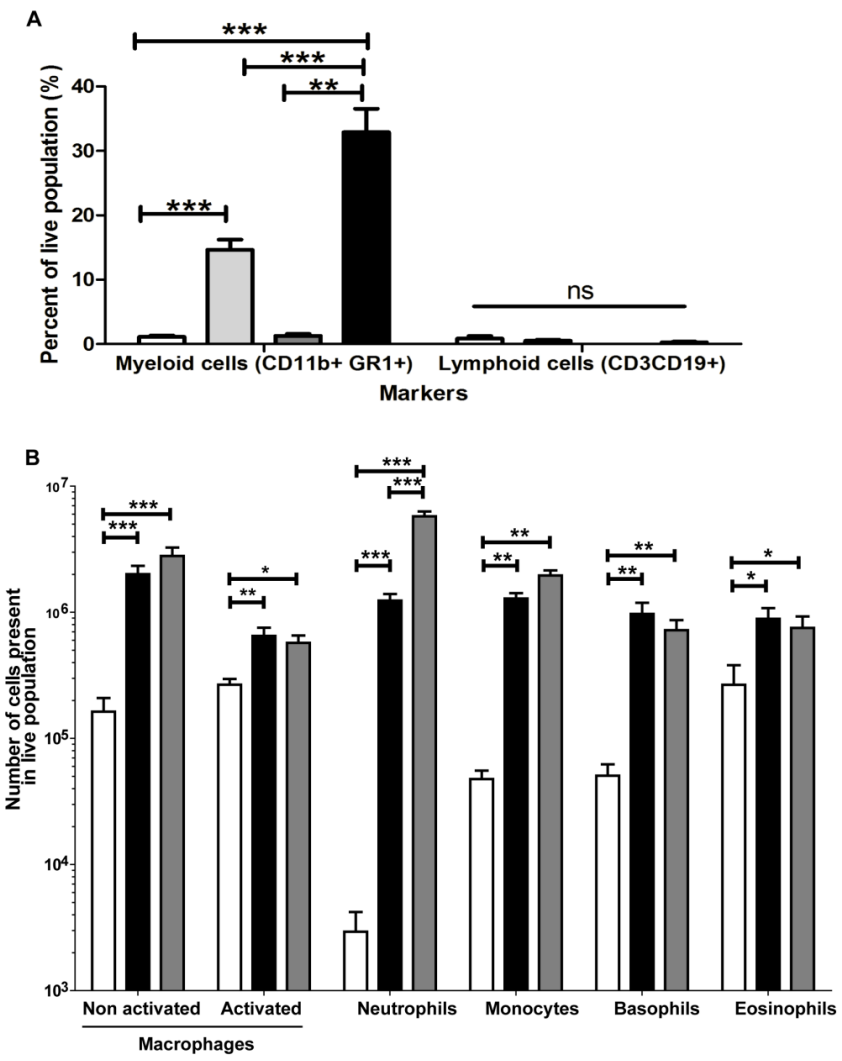

Figure 17: Neutrophils are important cellular infiltrates during enterococcal CAUTI.

Cellular infiltrates from non-implanted (white bars), implanted (black bars), or implanted and infected with *E. faecalis* OG1RF (gray bars) at 24hpi derived from flow cytometry analysis. Non-activated and activated macrophages ($CD11b^{+}F4/80^{+}MHCII^{-}$ and $CD11b^{+}F4/80^{+}MHCII^{+}$, respectively), neutrophils ($CD11b^{+}Ly6G^{hi}Ly6C^{lo}$), monocytes ($CD11b^{+}Ly6G^{-}Ly6C^{+}$), eosinophils ($SiglecF^{+}$), and basophils ($FccR1^{+}$). Data are the mean derived from at least two independent experiments with n=3-5/experiment/condition; Error bars represent SEM; *p<0.05, **p<0.005, ***p<0.0005 value by the Mann Whitney U test.

Part 2: Enterococcal biofilm formation and virulence following urinary implantation

E. faecalis *establishes persistent infections in the bladders and kidneys in an optimized murine model of CAUTI.* In order to study the pathophysiology of *E. faecalis*-mediated CAUTIs, mice were inoculated with 1-3.5x10^7 CFU of *E. faecalis* strain OG1RF by transurethral catheterization immediately following urinary implantation. In mock-infection studies with PBS, no bacteria were recovered from implants or organs (data not shown). At 24-hour post infection (hpi), OG1RF is recovered from the implants with a median of 9.76x10^5 CFU/ml (Fig. 19A). Bacterial titers on the abiotic surfaces increase significantly over the course of the infection (7 days) to a median of 8.64x10^6 CFU/ml (Fig. 19A). In implanted animals, OG1RF reaches a median titer of 3.2x10^6CFU/ml 24 hpi in the bladders, which is significantly higher (p=0.0011) than bacterial titers (2.7x10^3CFU/ml) recovered from the bladders of non-implanted animals (Fig. 19B). Similarly, bacterial titers from the kidneys increase significantly from 24 hpi to 7 dpi in implanted animals (Fig. 19C). OG1RF was readily cleared from the bladders and the kidneys within 48 hpi in the absence of implants (Fig. 19B-C). In addition, bacterial titers are significantly lower in tissues derived from animals that have lost their implants during the course of infection compared to tissues with retrieved implants (Fig. 20), with the exception of bladder titers at 48 hpi (p=0.0737) and kidneys titers at 72 hpi (p=0.130) (Fig. 21). These data suggest that the presence of the implants contributes to increased bacterial colonization and persistence in the urinary tract.

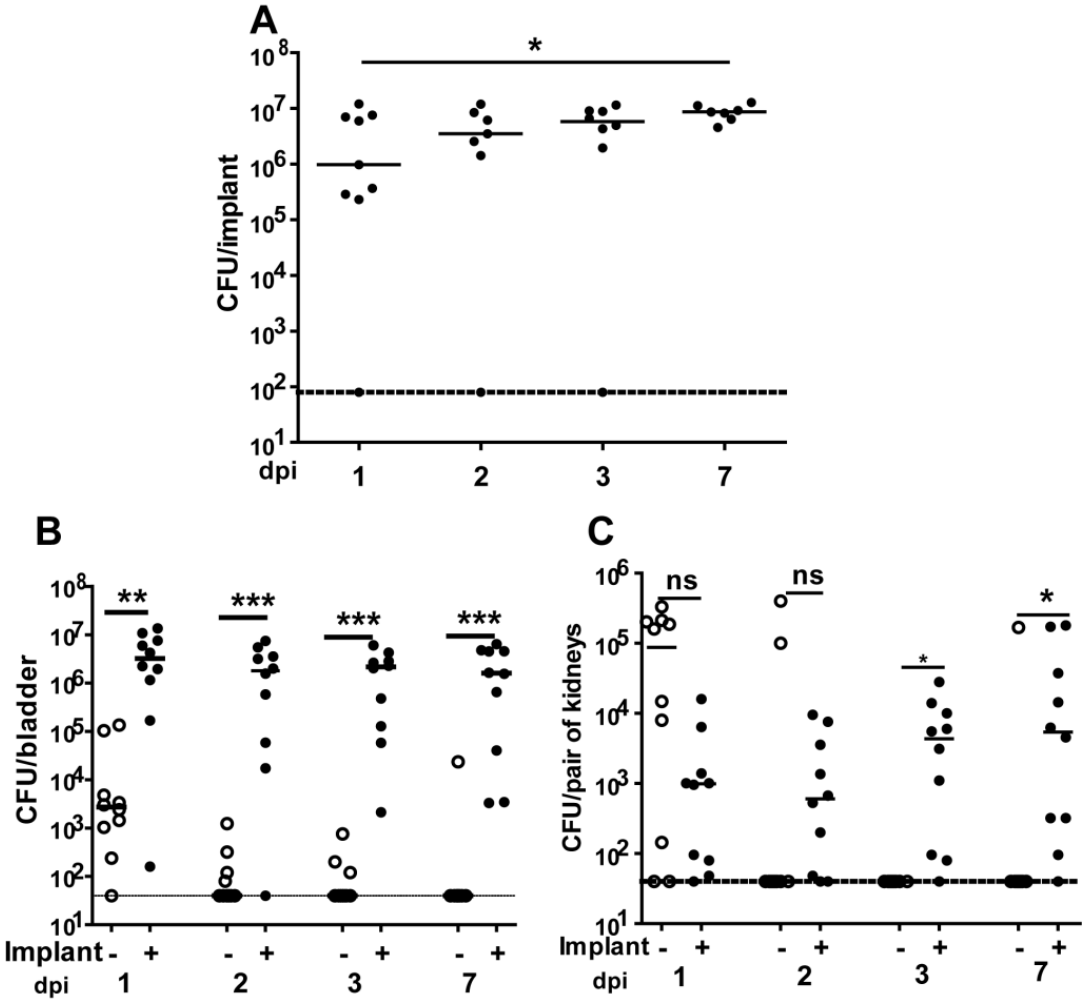

Figure 18: *E. faecalis* establishes persistent colonization of silicone implants, bladder, and kidneys in a murine model of CAUTI.

(A) Bacterial titers on logarithmic scale (log scale) from silicone implants retrieved from female C57Bl/6Ncr mice infected with OG1RF for 24, 48, 72 hpi, and 7 dpi. **(B-C)** Graphs represent bacterial titers from homogenized bladders **(B)** and kidneys **(C)** from non-implanted (○) or implanted (●) of animals infected with OG1RF for 24, 48, 72 hpi, or 7 dpi. Horizontal dashed lines represent the limit of detection for viable bacteria. Each symbol represents a mouse from two independent experiments with n=5/condition. The horizontal bars represent the median of each dataset; *p<0.05, **p<0.005, and ***p<0.0005 by the Mann Whitney U test.

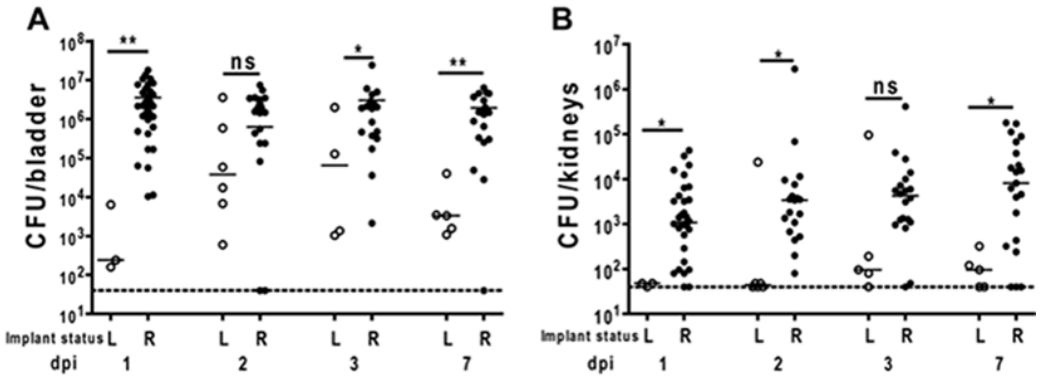

Figure 19: Implant loss from murine bladders correlates with lower bladder and kidney infection.

Graphs represent bacterial titers in log scale recovered from the bladders **(A)** and the kidneys **(B)** of animals that have lost the implants (L-○) or retained the implants (R-●) at the indicated time of sacrifice. Experiment was done at least 3 times with n=5/experiment. The horizontal dotted line depicts the limit of detection. The horizontal bar represents the median of each dataset, *p<0.05, **p<0.005, ns = p>0.05 by the Mann-Whitney U test.

E. faecalis *produces biofilm on the surface of the silicone implant* in vivo. To determine whether bacteria recovered from silicone implants are within biofilms, we examined 15 implants retrieved 72 hpi from murine bladders from three independent experiments by scanning electron microscopy (SEM). In mock-infection, the implants were coated on the outside and in the lumen with host factors found in the urine as previously reported[208] (data not shown). A similar film appears on 12 implants retrieved from animals infected with OG1RF. In approximately 70% of the silicone implants retrieved from infected animals, bacteria are readily observed in clumps both on the outer surface (Fig. 21A-B) or filling the entire lumen (Fig. 21C-F). The coccoid

microorganisms are embedded in a thick extracellular matrix (Fig. 21E-F). The reason for the absence of biofilms from the remaining implants is not certain, but could be attributed to sample processing prior to visualization since those animals also exhibited significantly enhanced infection in the bladders and kidneys. These findings indicate that enterococcal biofilms are produced *in vivo* on indwelling silicone material during the course of the infection.

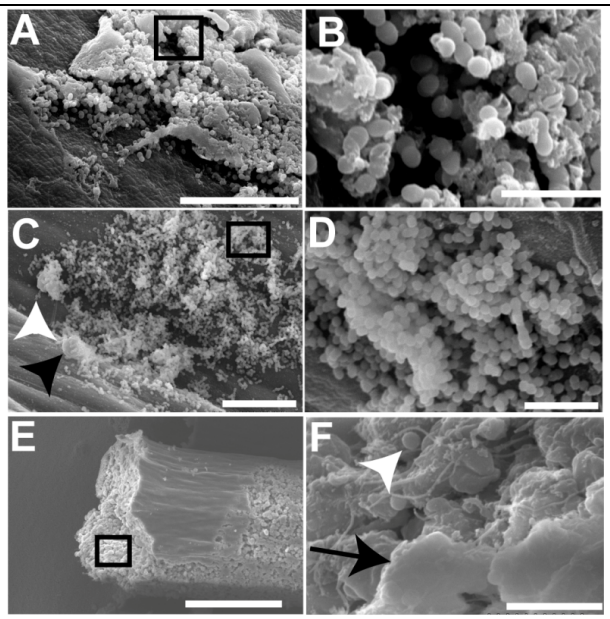

Figure 20: *E. faecalis* produces biofilms on the surface of silicone implants *in vivo*.

Representative scanning electron micrographs of silicone implants retrieved from murine bladders infected with OG1RF at 72 hpi showing bacterial biofilms on the outer surface **(A-B)** and the filling the lumen **(C-F)** of the silicone implant. Bacteria (white arrowhead) are often associated with host cells, indicated by black arrowhead in **(C)**. Both bacteria and host immune cells can also be found embedded in a thick extracellular material, indicated by arrow, in the lumen of the silicone implant 72 hpi **(E-F)**. Areas in the black box on **(A, C, E)** are magnified in **(B, D, F)**. Scale bars in μm: 20 **(A, C)**, 5 **(B, D, F)**, and 100 **(E)**.

E. faecalis ***enhances the inflammatory response in implanted animals.*** The histology of the uroepithelium of implanted bladders following infection with *E. faecalis* OG1RF is similar to that of mock-infected implanted animals in that the bladder remains edematous, distended, and sometimes hemorrhagic (Fig.15C and F). In contrast, non-implanted bladders infected with OG1RF show no major histological changes and appear similar to the bladders of mock-infected non-implanted controls (data not shown), in agreement with previous reports with other *E. faecalis* strains[190]. Immunolabeling of *E. faecalis* with antibody against the Lancefield streptococcal group D antigen in histology sections show bacterial staining in the bladder lumen of infected animals with implants (Fig. 15I). *E. faecalis* is also found in association with the surface of the uroplakin-positive and uroplakin-negative uroepithelium and occasionally found in the submucosal areas (Fig. 22). In contrast, very few bacteria were observed in the bladders of non-implanted animals 24 hpi (data not shown). No labeling was observed in mock-infected animals with or without implants (Fig. 15G and H), reaffirming the specificity of the antibody staining. Furthermore, enterococcal challenge does not affect murine bladder weight in presence or absence of urinary implants, as shown in Fig. 16D. The presence of *E. faecalis* slightly enhances the expression of IL-6, IL-12(p40), G-CSF, and KC and specifically induces the production of IL-1β and MIP-1α (back to non-implanted levels) in implanted animals compared to implanted mock-infected controls (Fig. 17). *E. faecalis* OG1RF infection of non-implanted animals results in no significant changes in the expression of the cytokines tested compared to mock infection (data not shown). Similar to uninfected implanted animals, $CD11b^{+}Gr1^{+}$ myeloid cells are the majority of cellular infiltrates at 24h in implanted animals with *E. faecalis* infection (Fig. 18A). They

account for approximately 30% of live populations in these animals, significantly higher than implanted controls in the absence of *E. faecalis* ($p<0.00001$). This increase in myeloid cells in presence of *E. faecalis* is primarily due to the recruitment of neutrophils with a significant increase of approximately 1 log over mock-infected implanted controls ($p<0.001$) (Fig.18B). Interestingly, similar numbers of macrophages, monocytes, basophils, and eosinophils are present in the bladders of implanted animals even in presence of *E. faecalis* (Fig. 18B), indicating that recruitment and activation of these immune cells occur specifically in response to the silicone implant. There are no significant changes in the number of dendritic cells ($CD11b^{+}CD11c^{+}MHCII^{+/-}$) present in implanted animals compared to non-implanted controls even in presence of *E. faecalis* (data not shown).

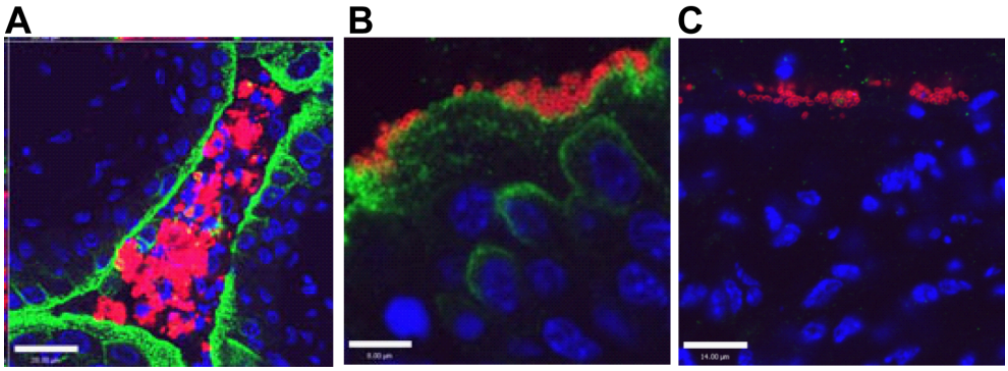

Figure 21: *E. faecalis* localization in implanted animals.

Representative CLSM images of bladder sections from implanted mice infected with OG1RF 24 and 72hpi immunolabeled with antibodies against uroplakin III (green), Lancefield Group D antigen (red), and counterstained with TOPRO-3 nuclear dye (blue). Bacteria are present in the bladder lumen **(A)**, uroepithelial surface **(B)**, and areas denuded of uroplakin staining **(C)**. Scale bar =28µm **(A)** 5µm **(B)** and 14 µm **(C)**.

Part 3: Relative contributions of known biofilm determinants to *E. faecalis* uropathogenesis

To assess the role of biofilm formation in enterococcal colonization of and persistence in the urinary tract, implanted animals were infected with OG1RF, a gelatinase-defective strain OG1X, or OG1RF isogenic in frame deletion mutants of known biofilm determinants.

E. faecalis *biofilm formation and virulence in the urinary tract occur independently of autolytic factors.* OG1X is a nitrosoguanidine-induced gelatinase (*gelE*)-defective mutant of the oral isolate OG1, the same parental strain as OG1RF[209]. Despite OG1X defect in gelatinase production, this strain produces biofilms to a similar extent as OG1RF on PVC coverslips in TSBG at 37ºC under static conditions as determined by crystal violet staining-based quantification (Fig. 23A). Notably, OG1X biofilms are also sensitive to DNaseI treatment under these conditions. Yet, when introduced in implanted animals, OG1X is recovered at significantly lower CFU than OG1RF from implants and bladders (p<0.0001) and is unable to ascend to the kidneys following urinary implantation 24hpi (Fig. 23B), indicating a severe attenuation in urovirulence. This observation led to the hypothesis that GelE may be necessary for virulence in the urinary tract. However, infection of implanted animals with an OG1RF isogenic *gelE*-deficient strain (OG1RFΔ*gelE*) causes similar colonization patterns as the parental strain (Fig. 23C), with slightly (albeit significant, p=0.0002) higher bacterial titers recovered from implants 24hpi. Furthermore, deletion of the major autolysis *atn* critical for DNA release during *in vitro* biofilm formation does not affect virulence in this murine model up to 7 days post infection (Fig. 23D and data not shown). Together, these data indicate that the autolytic

factors GelE and Atn (see chapter one) are dispensable for enterococcal virulence and OG1X may harbor additional mutations in genes required for urovirulence.

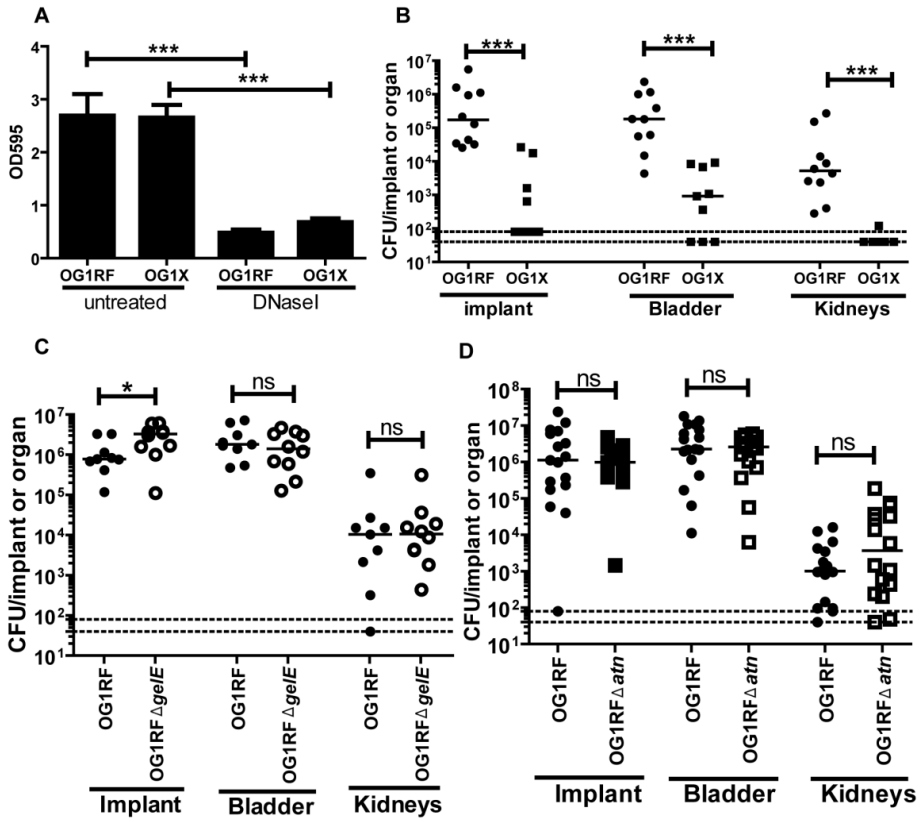

Figure 22: GelE and Atn are dispensable for enterococcal virulence in implanted animals.

(A) Comparison of OG1RF and OG1X biofilm formation on PVC coverslips in presence and absence of 5µg/ml of DNaseI in TSBG at 37ºC for 24h. n=3 independent experiments. Error bars=SEM **(B-D)** Graphs represent CFU enumeration of OG1RF, OG1X, OG1RF isogenic in-frame *gelE*- and *atn*-deficient mutants recovered from retrieved implants, bladders and kidneys 24 hpi with each strain. Horizontal dashed lines represent the limit of detection for viable bacteria. Each experiment was performed in duplicate n=4-5/strain/experiment. The horizontal bars represent the median of each dataset; *p<0.05; ***p<0.0005, ns = p>0.05 by the Mann Whitney U test.

Both enterococcal sortases contribute to implant and urinary tract colonization.

The housekeeping and pilus-associated sortases, SrtA and SrtC respectively, are important mediators of biofilm formation[106,109,210]. Additionally, deletion of *srtC* results in attenuation of *E. faecalis* virulence in a murine model of ascending UTIs[106]. In contrast to GelE and Atn, infection of implanted mice with *srtA*-deficient OG1RF strain (OG1RFΔ*srtA*) causes a 10^5-fold decrease in bacterial titers recovered from the implants (Fig. 24A) and an approximately 10^3 decrease in CFUs from both bladders and kidneys compared to wild type infection (Fig. 24B-C). This defect can be partially rescued by ectopic expression of a wild type copy of *srtA* in OG1RFΔ*srtA*. The complemented strain OG1RFΔ*srtA/srtA (*OG1RFΔ*srtA*c) is recovered at levels similar to wild type titers from the implants and organs (Fig. 24A-C). The lack of full complementation is not entirely understood, but may be due to plasmid loss during infection based on higher colony counts in the absence of selective antibiotic. A similar attenuation in virulence is observed upon deletion of *srtC* (Fig. 24A-C) whereby OG1RFΔ*srtC* is recovered at significantly lower titers from the implants (p=0.0017), the bladder, and the kidneys (p<0.0001 and p=0.0031, respectively) compared to wild type OG1RF. Similar findings are observed at 48 hpi (data not shown). Scanning electron micrographs reveal very few OG1RFΔ*srtA* cells present on the surface or in the lumen of retrieved implants 72 hpi in contrast to the complemented strain, the *atn*-deficient, or wild type strains that heavily colonize the implants (Fig. 24D). These results indicate that contrary to GelE and Atn, SrtA and SrtC or more specifically, SrtA-dependent substrate(s) and the enterococcal pilus (Ebp) (data not shown), are involved in biofilm production and necessary for full virulence in the urinary tract of implanted animals.

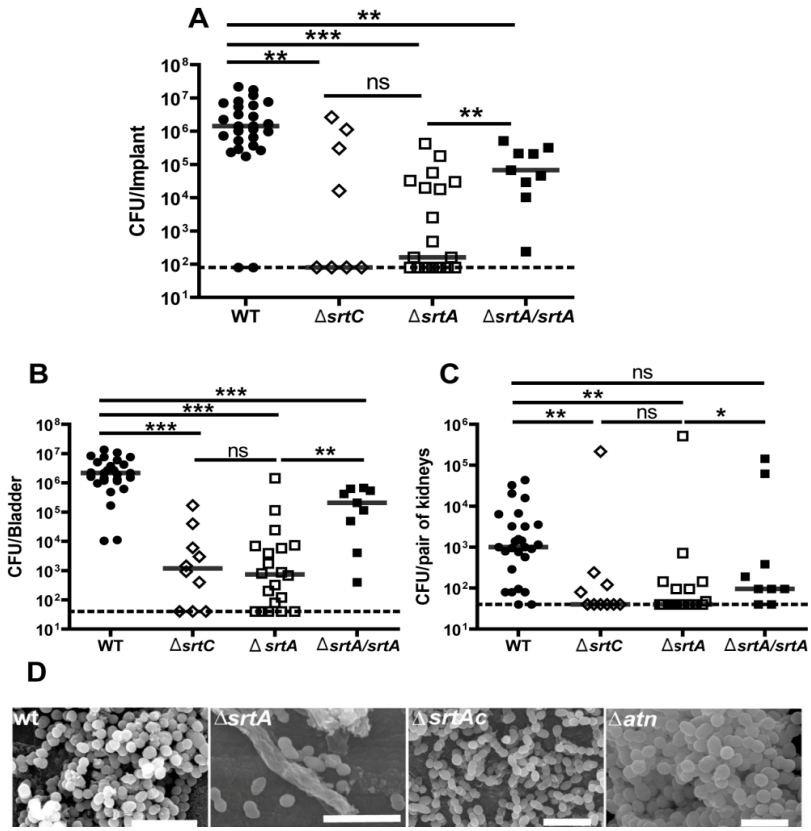

Figure 23: Sortase A is critical for implant colonization and virulence in the urinary tract. **(A-C)** Graphs represent bacterial titers from retrieved implants **(A),** homogenized bladders **(B),** and kidneys **(C)** from implanted mice infected for 24 hrs with OG1RFwt (●), OG1RFΔ*srtC*(◊) OG1RFΔ*srtA* (□), or OG1RFΔ*srtA*/*srtA* (■). Horizontal dashed lines represent the limit of detection for viable bacteria. Each symbol represents a mouse and each experiment was done at least twice with n=5/strain. The horizontal bars indicate the median of each dataset; *p<0.05, **p<0.005, ***p<0.0001, and ns for p>0.05 by the Mann Whitney U test. **(D)** Representative scanning electron micrographs of silicone implants retrieved from murine bladders 72 hpi with OG1RFwt, OG1RFΔ*srtA*, OG1RFΔ*srtA*/srtA (Δ*srtA*c)**,** and OG1RFΔ*atn*. Scale bars = 5µm for OG1RFwt, OG1RFΔ*srtA*, and OG1RFΔ*srtA*c; 2.5µm for OG1RFΔ*atn*.

Eep and AhrC contribute to* E. faecalis *pathogenesis in the urinary tract. The enterococcal *e*nhanced *e*xpression of *p*heromone (Eep) protein is a metalloprotease

involved in the processing of sex pheromones during conjugation[211,212]. RIVET screens identified the *eep* locus as one of several transcriptionally upregulated promoters during *E. faecalis* biofilm formation *in vitro* and *in vivo*[114,213]. Furthermore, in-frame deletion of *eep* in OG1RF in the absence of all conjugative plasmids results in significant virulence attenuation in a rabbit model of endocarditis, indicating this metalloprotease also has a role in virulence that is unrelated to conjugation/sex pheromones (Frank, K.L. *et al*, unpublished and personal communication). Preliminary evidence suggests that OG1RFΔ*eep* has altered biofilm architecture relative to the parental strain (Frank, K.L., *et al*, unpublished and personal communication). Similarly, transposon insertion in *ef0983,* a locus encoding a member of the ArgR family of transcription factors (*ahrC*), results in biofilm defects[109] and virulence attenuation in the rabbit model of endocarditis (Frank K. L. *et al*, unpublished and personal communication), involving AhrC as a biofilm-promoting and enterococcal virulence factor. The contributions of Eep and AhrC to enterococcal uropathogenesis were assessed using the murine model of foreign body-associated UTI. OG1RFΔ*eep* and OG1RFΩ*ef0983* were introduced in implanted animals and bacterial titers were assessed 24hpi and compared to the parental strain. Both strains are recovered at significantly lower titers from implants and kidneys compared to the parental strain ($p<0.05$ in all cases) (Fig. 25). However, whereas no defects were observed in bladder colonization with OG1RFΔ*eep* ($p>0.05$), OG1RFΩ*ef0983* was significantly attenuated in the bladder ($p=0.0003$) as shown on (Fig. 25) (Frank, K.L., Guiton, P.S., *et al*, manuscript in preparation). Together, these findings indicate that both genes contribute to urovirulence and furthermore underscore the usefulness of the optimized murine model of CAUTI for the identification of novel virulence factors

specifically involved in enterococcal colonization of the urinary tract that are also critical for other enterococcal diseases.

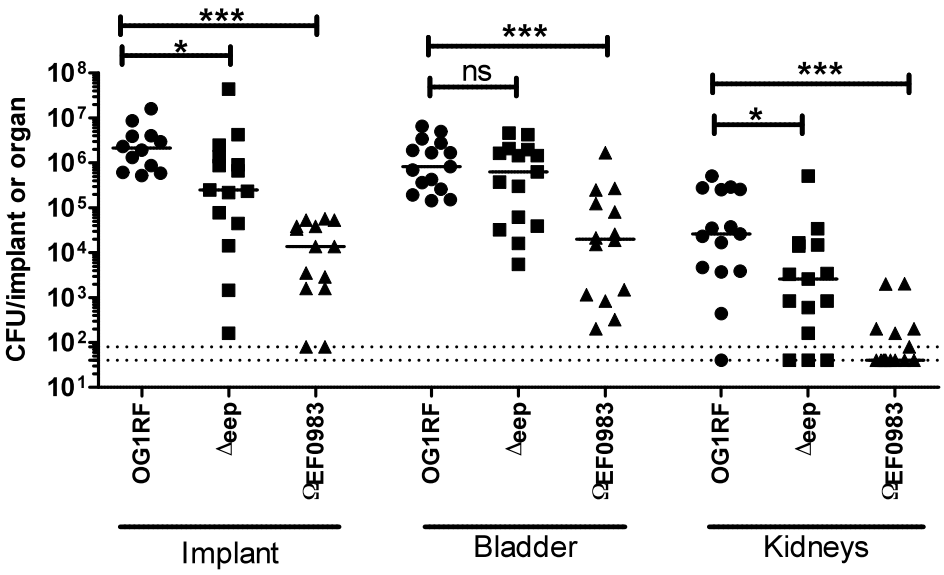

Figure 24: Eep and AhrC promote enterococcal uropathogenesis

Graph represents bacterial titers from retrieved implants, homogenized bladders, and kidneys from implanted mice infected for 24 hrs with OG1RF (●), OG1RFΔ*eep* (■) OG1RF*ΩEF0983* (▲). Horizontal dashed lines represent the limit of detection for viable bacteria. Each symbol represents a mouse and each experiment was done at least twice with n=5/strain. The horizontal bars indicate the median of each dataset; **p*<0.05, ****p*<0.0005, and ns for *p*>0.05 by the Mann Whitney U test.

Part 4. Role of implant-mediated inflammation during *E. faecalis* pathogenesis in the urinary tract

Induction of bladder inflammation is not sufficient for the establishment of successful* E. faecalis *infection without a foreign body. To address the effects of implant-mediated inflammation on enterococcal infection, animals were implanted for 24h prior to infection

with *E. faecalis* OG1RF strain and bacterial titers from the implant and organs were compared to titers recovered from animals implanted and simultaneously infected. As shown on Fig. 26A, *E. faecalis* is able to colonize the implants and organs of pre-implanted animals to similar levels as the control group, indicating that *E. faecalis* is resistant to the implant-induced inflammatory response, which does not enhance nor prevent enterococcal colonization.

Furthermore, to assess whether any type of cystitis predisposes the bladder to *E. faecalis* infection in the absence of foreign body, murine hemorrhagic cystitis was induced via treatment with cyclophosphamide (CYP) (150kg/mg, i.p.). CYP-induced cystitis is a well-characterized model of bladder inflammation and involves among others, the induction of the neurogenic inflammatory pathway described above[198,214]. Even though implant-mediated inflammation occurs independently of the neurogenic inflammatory pathway, it shares some commonalities with CYP-induced cystitis such as bladder wall edema, mucosal damage, and neutrophil infiltration[203,204,215,216] (Fig. 26B). *E. faecalis* introduced in non-implanted CYP-treated animals are rapidly cleared from the urinary tract similar to saline-treated non-implanted controls at 6hpi (Fig. 26C). By 24hpi, bacteria are recovered at very low levels (10^2-10^3 CFU/ml in organs) from both experimental groups compared to implanted animals whose organs remain colonized at very high titers (10^5-10^6CFU/ml in organs as well as implant; data not shown). Together, these findings indicate that the inflammatory state of the bladder is not sufficient to promote *E. faecalis* infection of the urinary tract in the absence of a foreign body.

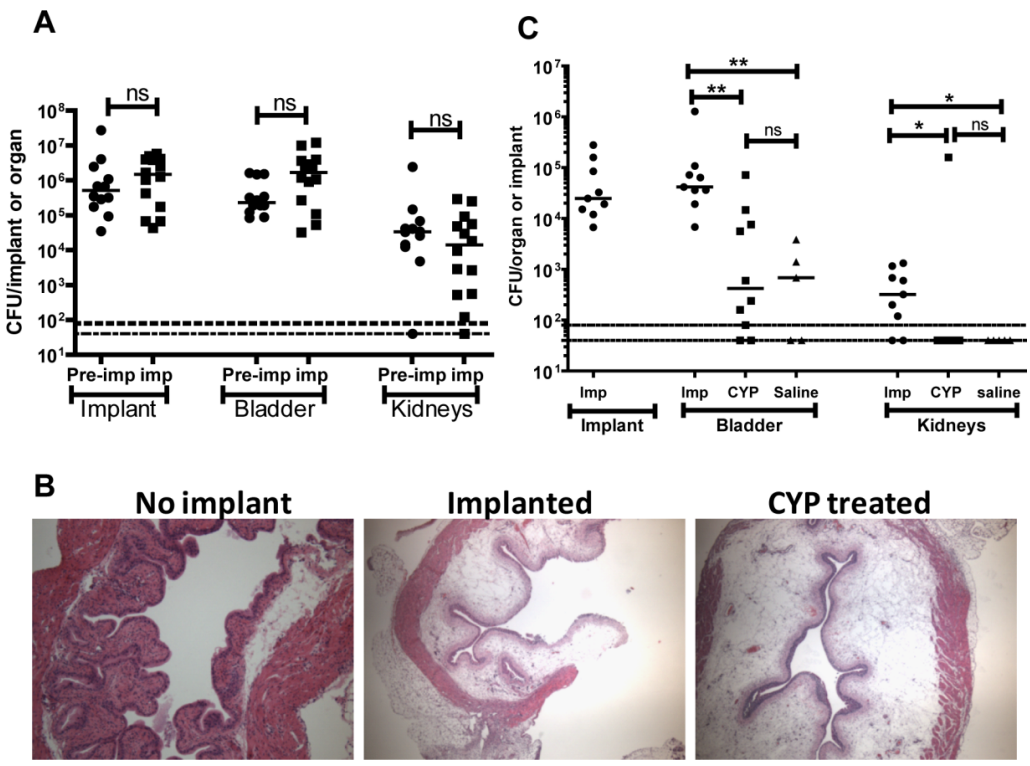

Figure 25: Inflammation is not sufficient enterococcal urinary tract colonization

(A) CFU enumeration of OG1RF from implant, homogenized bladders and kidneys of animals pre-implanted (Pre-imp) 24h prior to or implanted (imp) on the day of bacterial challenge. **(B)** H/E staining of bladder sections of 6 h cyclophosphamide (CYP)-treated or implanted mice observed under light microscope, magnification 20X. Experiment was performed in duplicate with n≥3-5/group. **(C)** Graph represents OG1RF titers at 6hpi from retrieved implants, homogenized bladders, and kidneys from non-implanted CYP- or saline-treated and implanted (Imp) animals. For **(A)** and **(C)** The horizontal bars indicate the median of each dataset. Two independent experiments performed with n=5-10/condition/experiment. *p<0.05, **p<0.005 value by the Mann Whitney U test.

Glucocorticoid treatment partially inhibits implant-induced inflammation and enhances* E. faecalis *urovirulence. Since induction of bladder inflammation does not alter the pathogenesis of *E. faecalis*, further experiments were performed in implanted

animals in presence of an anti-inflammatory and immunosuppressive agent to further assess the role of implant-induced inflammation during enterococcal CAUTI. Dexamethasone, a well-characterized glucocorticoid, was administered in mice at a concentration of 10mg/kg, i.p. 30 min prior to implantation. This treatment inhibits the increase in bladder weight and the onset of edema up to 9h post implantation compared to saline treated controls (Fig. 27A) and causes similar degree of vascular permeability relative to non-implanted animals at 6hpi (Fig. 16C). However, by 12h post implantation, the bladders of dexamethasone-treated animals were as edematous and inflamed as saline-treated implanted controls (Fig. 27B). Supplemental dosages of dexamethasone administered 30 min prior to implantation and at 9h post implantation did not prevent edema at 24h post implantation (data not shown). Interestingly, preliminary cytokine profiling at 24h post implantation reveals that dexamethasone-treated and implanted animals have significantly reduced G-CSF, KC, and interleukins 1β, 6, 12p40, and 17, compared to saline-treated implanted controls (Fig. 28B). These soluble inflammatory markers are upregulated at least two-fold in saline-treated implanted animals over non-implanted mock-infected controls following urinary implantation (Fig. 28B). Furthermore, dexamethasone-treatment significantly ($p<0.05$) reduced the infiltration of monocytes, basophils and eosinophils in the bladder of implanted animals compared to implanted controls at 24h post implantation (data not shown). Together, these data indicate that activation of glucocorticoid-sensitive immune pathways contributes to the onset of bladder wall edema, vascular permeability, pro-inflammatory cytokine expression, and cellular recruitment following urinary implantation.

The effects of dexamethasone treatment on enterococcal virulence in implanted animals were assessed at 6h and 24hpi. As shown in Fig. 28A, enterococcal titers were ~1 log significantly higher (p=0.0424) on implants recovered from dexamethasone-treated animals compared to saline-treated implanted controls at 6hpi. No significant difference in bacterial titers was observed in the bladder and kidneys between the two groups. By 24hpi, *E. faecalis* recovery from implants was similar in dexamethasone- and saline-treated animals (Fig. 28A). Interestingly, preliminary evidence indicate that enterococcal infection in dexamethasone-treated animals restores the production of pro-inflammatory markers, including IL-6, (Fig. 28B) and myeloid cell recruitment back to the levels observed in saline-treated implanted controls (data not shown), suggesting that *E. faecalis* infection induces a dexamethasone-independent immune response that is ineffective at preventing and clearing *E. faecalis* from the urinary tract in the presence of a silicone implant.

Implanted IL-6- and TLR-2-defective mice are susceptible to enterococcal infections similar to their wild type littermates. Since IL6 is the most up-regulated pro-inflammatory cytokine in response to urinary implantation, its contribution to the inflammatory response during *E. faecalis* infection was determined following OG1RF inoculation of implanted IL6-deficient mice. *E. faecalis* is recovered from implants, bladder, and kidneys of IL6-deficient animals at similar levels as their littermate wild type controls (Fig. 29). Notably, preliminary data indicate that the absence of this cytokine does not affect the overall inflammatory response at 24hpi as assessed by cytokine profiling and flow cytometry analysis of wild type and IL-6-deficient animals with or without implants in presence or absence of *E. faecalis* (data not shown).

Together, although IL-6 is highly induced following urinary implantation, it is not a critical mediator of the inflammatory response in implanted animals with or without *E. faecalis* infections.

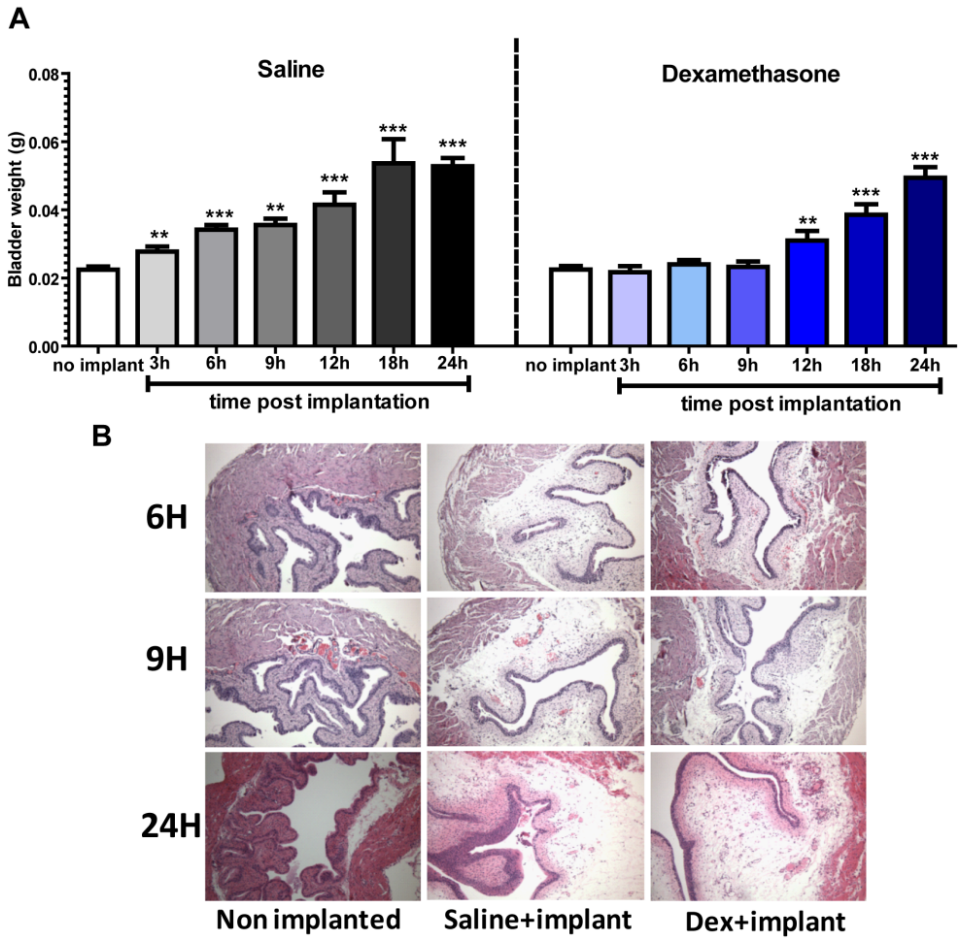

Figure 26: Dexamethasone treatment delays onset of bladder edema.

(A) Bladder weights of female C57Bl/6Ncr at the indicated time points treated with saline or dexamethasone (10mg/kg, i.p.) 30 min prior to implantation. White bars from the same dataset represent weight of non-implanted animals' bladders. Experiment was done at least twice with n=5/experiment/time point, except at 12 and 18h (experiment performed once). Error bar = SEM. $**p<0.005$, $***p<0.005$ by the Mann-Whitney U test. **(B)** H/E staining of bladder sections obtained at the indicated time points from non-implanted and implanted animals treated with saline or dexamethasone (10mg/kg, i.p.) observed under light microscope at 40X magnification.

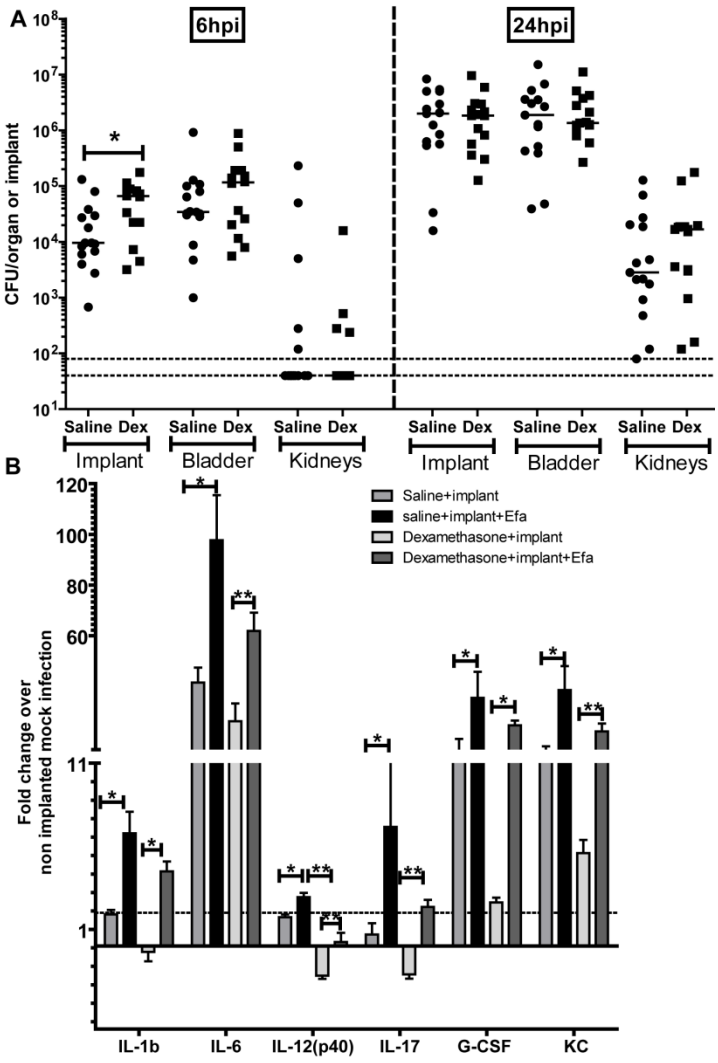

Figure 27: Dexamethasone treatment enhances early enterococcal implant colonization.

(A) Graph represents OG1RF titers at 6 and 24hpi from retrieved implants, homogenized bladders, and kidneys from saline or dexamethasone-treated implanted mice. The horizontal bars indicate the median of each dataset from at least two independent experiments with n=5-10/condition/experiment. The horizontal dashed lines represent the limit of detection. **(B)** Graph represents bladder cytokines with at least two fold differential expression relative to uninfected non-implanted animals at 24 hpi of non-implanted and saline- or dexamethasone-treated implanted animals with or without OG1RF from at least two independent experiments n=2-3/condition/experiment. Error bars = SEM. *p<0.05, **p<0.005 by the Mann Whitney U test.

Given that myeloid cells, the primary responders to silicone implants, are critical for the host pathogen recognition via expression of Toll-like receptors[217] and TLR2 recognizes lipoteichoic acid on the surface of Gram positive bacteria[218], the contribution of TLR-2 was specifically assessed during *E. faecalis* infection. Implanted TLR-2-defective mice are as susceptible to *E. faecalis* colonization as their wild type littermates (Fig. 29). Preliminary confirmatory evidence was obtained from enterococcal infections of mice deficient in the TLR adaptors MyD88 and TRIF, independently (data not shown). Therefore, these findings suggest that the Toll-like receptor pathway is not critical in the host immune recognition of *E. faecalis*, in agreement with the limited activation of macrophages even in the presence of high levels of *E. faecalis*.

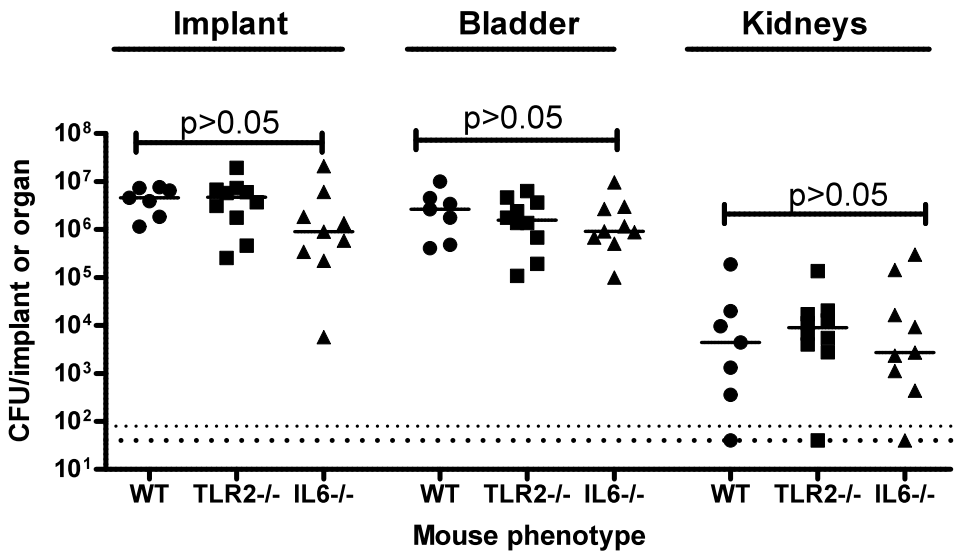

Figure 28: Defects in IL6 and TLR2 do not alter *E. faecalis* uropathogenesis.

Graph represents OG1RF titers at 6 and 24hpi from retrieved implants, homogenized bladders, and kidneys of WT, TLR-2- and IL-6- deficient mice. The horizontal bars indicate the median of each dataset from at least two independent experiments with n=5/condition/experiment. The horizontal dashed lines represent the limit of detection.

Neutrophil recruitment contributes to anti-enterococcal responses. Neutrophils are the major immune cells recruited in response to *E. faecalis* infection in the urinary tract of implanted animals (Fig. 18B). To assess the contribution of neutrophils to the inflammatory response during *E. faecalis* infection, mice were rendered neutropenic following anti-ly6G treatment[219] administered i.p. 3 days and 1 day prior to implantation and bacterial challenge. At 24hpi, there was an approximately 1 to 1.5 log increase in CFU recovered from implants and organs of neutrophil-depleted animals compared to isotype-control antibody-treated implanted animals ($p<0.05$ in all cases) (Fig. 30). Together, these findings indicate that neutrophils are important for controlling enterococcal colonization of the urinary tract during CAUTI.

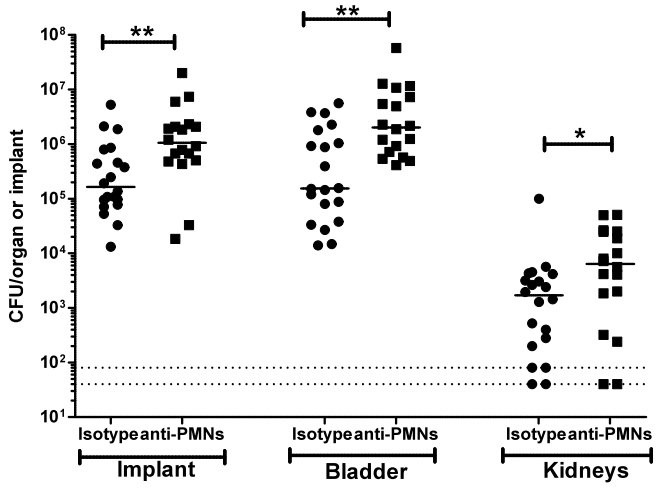

Figure 29: Neutrophil depletion enhances enterococcal CAUTI.

Graph represents OG1RF titers at 24hpi on implants, in homogenized bladders and kidneys of implanted isotype IgG control- (Isotype) and anti-Ly6G- (anti-PMNs) treated mice. The horizontal bars indicate the median of each dataset from two independent experiments with n=10/condition/experiment. The horizontal dashed lines represent the limit of detection for implant and organs. *$p<0.05$, **$p<0.005$ value by the Mann Whitney U test.

DISCUSSION

Enterococci, normally commensal bacteria of the human oral cavity and gastrointestinal tract, have become important opportunistic pathogens in hospital settings. While not historically regarded as an uropathogen in uncomplicated community acquired cystitis, *E. faecalis* is now considered a major agent of hospital-acquired UTIs[56] where catheterization and indwelling medical devices can negatively impact patients' defenses against pathogens[56]. However, the lack of a robust animal model of enterococcal UTI has impeded the study of this pathogen within the urinary tract. In previously established models of ascending UTIs, *E. faecalis* fails to establish persistent bladder colonization and primarily displays tropism to the kidneys before rapid clearance by the host[105,187-191]. The absence of persistent infections in these murine models reflects the profile of *E. faecalis* in community-acquired UTIs where this bacterium represents less than 5% of clinical isolates[220]. As an opportunistic pathogen, *E. faecalis* requires alterations in bladder homeostasis following urinary catheterization in order to achieve successful infection of the urinary tract.

In this study, the pathophysiology of *E. faecalis*-mediated CAUTIs was characterized using a murine model of foreign body-associated UTI modified from previous studies[29,194]. In this murine model, *E. faecalis* colonizes and persists within the urinary tract at very high titers. The striking difference in enterococcal bladder colonization and virulence from previous models is a direct consequence of implantation, which elicits major histological and immunological changes in the bladder similar to those observed in spinal cord injured and post-surgical patients with indwelling urinary catheters[25-27,30]. The importance of the bladder implant during *E. faecalis* uropathogenesis

was made evident from the efficient bacterial clearance and the significantly lower titers recovered from the urinary tract in the absence and/or with the loss of implants, even in inflamed bladders following cyclophosphamide treatments. This is because the implant provides an abiotic surface for enterococcal biofilm production within the bladder.

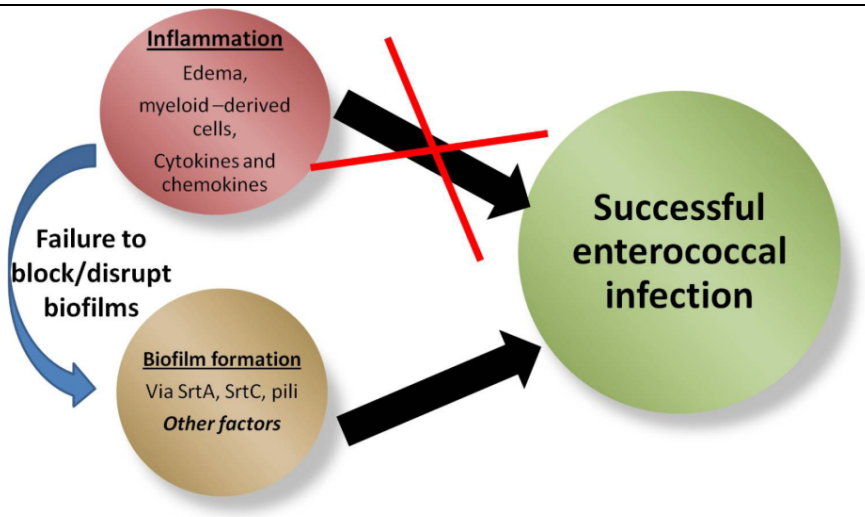

Figure 30: Model of enterococcal uropathogenesis in the context of urinary catheterization.

The insertion and presence of a foreign body within the bladder induces an acute inflammatory response involving edema, neutrophilia, and the production of pro-inflammatory cytokines and chemokines. This inflammatory response does not promote E. faecalis infection. It is postulated that the failure of the host to block and disrupt enterococcal biofilms produced on the surface of the foreign body leads to successful enterococcal infections. Biofilm formation, involving the actions of SrtA and SrtC, as well as other known and unknown biofilm determinants is critical for *E. faecalis* uropathogenesis.

Biofilms are central to the pathogenesis of chronic infections, including CAUTIs[38,40,112]. Enterococcal biofilms on the surface of implants is essential for the

establishment of persistent enterococcal urovirulence as demonstrated by virulence attenuation of strains with mutations in genes encoding known and yet to be identified biofilm determinants. Bacteria within biofilms, protected from the host immune assaults[49], may continuously seed the bladder preventing effective clearance by the host and leading to persistent cystitis and pyelonephritis. It is important to note that not all known *in vitro* biofilm-promoting or virulence factors are involved in *E. faecalis* pathogenesis within the urinary tract as shown in the present study. Specifically, autolytic factors, such as GelE and Atn, are important mediators of DNA release during biofilm growth *in vitro*[103,104,121,127,210] and GelE a major virulence factor in animals models of endocarditis[221]. Deletions of both *atn* and *gelE* do not alter enterococcal biofilm formation on the implants nor virulence in the urinary tract. The ability of *E. faecalis* to colonize the implants and establish persistent infections in the absence of both Atn and GelE argues that extracellular DNA may not be an important contributor of the extracellular matrix of enterococcal biofilms *in vivo* in the urinary tract or its release may occur in an Atn/GelE-independent manner. Furthermore, the ability of the *E. faecalis* *gelE*-defective OG1X strain to produce DNA-dependent biofilms *in vitro*, but yet unable to colonize the implants and organs in the urinary tract, further supports the claim that GelE is not a critical virulence factor for enterococcal uropathogenesis. However, since OG1X was derived from chemical mutagenesis[209], it is likely that additional mutations that may compensate for the loss of GelE production during DNA-dependent biofilms and/or are necessary for *E. faecalis* uropathogenesis *in vivo*. Consistent with these findings was the observation that gelatinase is not expressed in 71/163 clinical isolates[172] of *E. faecalis* and the presence of *gelE* does not correlate with gelatinase expression[192] or

their ability to produce biofilms *in vitro*[172]. However, a recent study by Arciola *et al* found a correlation with a high GelE phenotype and the ability to form biofilms from orthopedic implant infections[222], indicating that the factors utilized by enterococci in forming biofilms may depend on substrate and/or site of infection. Identification of the major components of the extracellular matrix of *in vivo* enterococcal biofilms and the single nucleotide polymorphisms (SNPs) between OG1RF and OG1X will lead to a better understanding of enterococcal CAUTIs and will be of valuable interest for designing therapeutics that promote the prevention and disruption of these structures.

While GelE and Atn are dispensable for *E. faecalis* pathogenesis, the enterococcal *e*nhanced *e*xpression of *p*heromone (Eep) metalloprotease, the transcriptional regulator AhrC, and the enterococcal sortases are important mediators of full virulence in implanted animals. In particular, sortases A and C are well-established biofilm-promoting factors in *E. faecalis*[106,109,210]. Recent studies have shown that sortases, which are transpeptidases that anchor LPXTG-containing surface proteins to the cell wall of Gram-positive bacteria[143], plays important roles in *in vitro* biofilm development[106,109,210]. Deletions of both *srtA* and *srtC* in *E. faecalis* prevent adherence and subsequent biofilm growth on abiotic surfaces[210]. Although Kemp *et al* reported that disruption of *srtA,* unlike *srtC*, did not significantly affect virulence in a murine model of ascending UTI[106], results from the murine model of enterococcal CAUTI reveal that SrtA contributes to *E. faecalis* virulence in the urinary tract. The *srtA*-deficient mutant is unable to colonize the implants *in vivo* and is significantly attenuated in CAUTI. Similar results were obtained following infection of implanted animals with a *srtC*-deficient mutant. These findings further underscore the importance of biofilm production during *E. faecalis* pathogenesis

and strongly suggest that SrtA-dependent substrates and the enterococcal *e*ndocarditis- and *b*iofilm associated *p*ili (ebp) found in most clinical isolates of *E. faecalis* and several *E. faecium* strains[167,223] are involved in attachment to abiotic surfaces and/or to abraded uroepithelium *in vivo* during the establishment of CAUTI. Evidence obtained in collaboration with Hailyn V. Nielsen at Washington University in St. Louis who generated a deletion mutant of the *ebp* operon in OG1RF demonstrates a function for Ebp in virulence during CAUTI (Nielsen, H.V., Guiton, P.S., *et al*, manuscript in preparation), in agreement with previous reports that mutations in this operon significantly reduces biofilm formation and attenuates virulence in a murine model of ascending UTIs in *E. faecalis* and *E. faecium*[107,145,167,223]. It will be of particular interest to understand the molecular mechanisms underlying the role of Epb during CAUTI and identify additional SrtA-dependent substrates[116] critical for urovirulence as they represent attractive targets for drug development given their ubiquitous nature and important functions in virulence [116,164]. For example, recently characterized LPxTG motif-containing proteins, EF3314 and SrgA, contribute to enterococcal biofilm formation[169,224]. EF3314 is also involved in *E. faecalis* adherence to host cells and survival within macrophages and *Caenorhabditis elegans*[169]. Furthermore, surface proteins known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which include putative SrtA substrates, have been identified in *E. faecium* and *E. faecalis*[225-227]. MSCRAMMs, such as enterococcal collagen-binding adhesin *ace*[165,228] or the SagA-like SalA and SalB proteins[229], can bind to host cells via interactions with extracellular matrix components like collagen and fibrinogen. Several proteins from this family have been implicated in enterococcal virulence properties, biofilm formation, endocarditis and UTIs[91,167,230,231].

The disruption of urothelial surfaces, as a result of urinary catheterization, may expose receptors that SrtA-dependent cell surface proteins, such as MSCRAMMs, can recognize to promote attachment, biofilm formation and persistent colonization.

Additionally, the findings in this report helps unravel critical aspects of the interplay between inflammation and enterococcal colonization, providing new details of the molecular mechanisms leading to implant-mediated inflammatory response and its role on the outcome of *E. faecalis* infection. The bladder responses to urinary catheterization are characterized by severe uroepithelial damage, the onset of bladder wall edema from increased vascular permeability, and upregulation of inducible nitric oxide synthase (preliminary data not shown). The presence of the implant stimulates the production of pro-inflammatory cytokines, including IL-6 and KC (IL-8 or CXCL1) previously shown to be induced following catheter-mediated abrasions of bladder epithelial cells *in vitro*[232], and G-CSF. In contrast, GM-CSF and MIP-1α (CCL3) expression was decreased following implantation. The presence of *E. faecalis* in implanted animals, albeit at very high titers, slightly enhances the inflammatory response with a specific increase in the production of IL-1β, GM-CSF and MIP-1α (the latter back to non-implanted, uninfected control levels) during CAUTI. These cytokines are produced by epithelial cells, neutrophils and other immune cells and are important mediators of the inflammatory response to mucosal injuries and microbial infections[233-238]. In particular, IL-1β was shown to be released during UPEC-mediated acute cystitis[239]. This cytokine profile of implanted bladders is in agreement with the identity of the myeloid cell infiltrates primarily in response to urinary implantation. Neutrophils, whose

presence is doubled in *E. faecalis* infected implanted animals, are the primary responders followed by monocytes, macrophages, basophils, and some eosinophils.

All the above immune characteristics, from edema to neutrophilia, are associated with activation of the neurogenic inflammatory pathway in various experimental models of cystitis, including cyclophosphamide-induced hemorrhagic cystitis[214]. This is an inflammatory response triggered by the release of proinflammatory neuropeptides and activation of surface receptors, including NK1R, on the surface of sensory neurons[197]. However, NK1R and α1-adrenoreceptor-mediated neurogenic inflammatory responses are not major contributors of implant-induced cystitis in mice since treatment with specific NK1R antagonists and α1-adrenoreceptor as well as iNOS inhibitors did not prevent plasma protein leakage and edema as was previously shown in bladder cystitis or other experimental models involving activation of the neurogenic pathway[204,206,240]. This is further supported by preliminary evidence from implantation of *cKit*-deficient mutant mice revealing that mast cells, important cellular components of the neurogenic pathway[198,241,242], are not major inducers of edema and vascular permeability following urinary implantation (data not shown). Although the contribution of other factors involved in the neurogenic inflammatory response, including bradykinins and NK2 receptors, need to be assessed, identifying the effects of urinary implantation on factors involved in vascular permeability, such as calcium channels, calveolin, RhoGTPases, sphingosine kinases (SPHK1) and protein tyrosine phosphatases (SHP2)[243], may shed light into the mechanisms underlying the onset of bladder wall edema following catheterization.

In contrast to studies with inhibitors of the neurogenic inflammatory response, glucocorticoid treatment delays the onset of implant-associated edema and partially decreases cytokine production and cellular recruitment following urinary catheterization, thus implicating glucocorticoid-responsive inflammatory pathways in the immune response during the early stages of urinary implantation. Synthetic glucocorticoids such as dexamethasone are the most effective anti-inflammatory agents used to date for the treatment of chronic inflammatory diseases[244]. They are both anti-inflammatory and immunosuppressive molecules whose mechanisms of action involve in part transcriptional regulation via interaction and activation of glucocorticoid receptors (GR) in the host cytoplasm as well as post transcriptional and translational regulation of a myriad of genes encoding proteins for cellular and immune processes[244,245]. Glucocorticoids are known inhibitors of inflammatory processes mediated by interleukins (1β, 2, 6, and 8) and other pro-inflammatory cytokines such as (GM-CSF and TNF-α), cyclo-oxygenases (particularly COX-2), and iNOS. The unresponsiveness to dexamethasone observed in the later stages of urinary implantation has previously been reported in patients suffering from other inflammatory diseases[246], including asthma[247], inclusion body myositis (IBM)[248], and nephrotic syndrome (NS) in children[249]. In the case of the implanted bladders, glucocorticoid refractoriness could be attributed to the ongoing exposure to the foreign body[250], the high levels of neutrophils in the bladder which have been previously associated with corticosteroid-resistant asthma[250], a significant reduction in glucorticoid receptors[251] due to increased urothelial exfoliation, or other cellular and immunological pathways that can circumvent the effects of dexamethasone[250]. These may include an increased expression of the dominant negative

form of the glucorticoid receptors (GRβ) on immune cells such as neutrophils and macrophages rendering them insensitive to dexamethasone treatment[252,253], upregulation of certain cytokines including IL-2, IL-4, and IL-13, or from activation of the mitogen-activated protein kinase (MAPK) signaling pathways[250]. Notably, the suppression of the early phase of implant-mediated cystitis following glucocorticoid treatment or iNOS inhibition (data not shown) led to a significant increase in enterococcal implant colonization. Remarkably, enterococcal infection in dexamethasone-treated animals induces an immune response similar to that elicited by the implants in uninfected saline-treated controls. These findings suggest that *E. faecalis* possesses immune evasion mechanisms that allow its survival in the face of this glucocorticoid-resistant immune response and that implant-mediated bladder inflammation as in the case of pre-implanted animals did not alter the outcome of infection. The acute inflammatory response induced by the implant may even alter or impair the host response to bacteria, as was demonstrated for *E. faecium* peritonitis following treatment with turpentine or casein prior to bacterial challenge[254].

E. faecalis colonization is also significantly increased in the bladders of neutropenic mice following urinary implantation, corroborating previous reports of that neutrophils are important mediators of the anti-enterococcal host response in humans and other animal models of infections[236,255-257]. Previous studies demonstrated that *E. faecalis* and *E. faecium* isolated from saliva and root canals are efficiently killed by neutrophils recruited to the site of infection[258] and that TLR-2 is involved in the immune response against *E. faecium*[259]. However, the immune functions of neutrophils during *E. faecalis* infections of the urinary tract occur in a TLR-2 and IL-6 independent manner as infection

of implanted animals deficient in these immune modulators did not alter the outcome of infection. Further research is required to establish the contribution of macrophages and other immune cells, the toll like receptors, IL8, and G-CSF signaling pathways in the host immune response to enterococcal CAUTI.

Despite the role of neutrophils in controlling *E. faecalis* infection, this bacterium is still able to colonize the urinary tract of implanted immunocompetent mice, implying the presence of potential mechanisms to help *E. faecalis* avoid and/or resistant neutrophil killing. Recent studies have demonstrated that the cell wall anchored pheromone-inducible *a*ggregation *s*ubstance (AS) and the *e*nterococcal *p*olysaccharide *a*ntigen (Epa) in *E. faecalis* are involved in resistance to neutrophil-mediated killing[260-262]. However, the *E. faecalis* OG1RF strain used in the present study does not bear AS, arguing for alternative mechanisms of immune evasion, such as the downregulation of integrin 4 expression of the surface of neutrophils[263], alteration of the neutrophil properties rendering them non responsive to bacterial infections as is the case during enterococcal sepsis in thermally injured patients and mice[236,256,257] or survival within immune cells such as macrophages which is a well characterized virulence attribute of enterococci [186,264-271]. Together, these findings indicate that the inflammatory response to the urinary implant can be deleterious to *E. faecalis*, but is inefficient at controlling bacterial proliferation and colonization over time. Furthermore, this report is in accord with epidemiological reports of severe enterococcal infections increasingly occurring in immunosuppressed and immunocompromised patients[272-275].

In addition to promoting persistent enterococcal cystitis, the presence of the silicone implants in the bladder allows *E. faecalis* to gradually and successfully ascend to

the kidneys and establish renal colonization in implanted animals. The onset of acute pyelonephritis has been described in post-mortem studies of the elderly with indwelling catheters at the time of death[276]. In the clinical setting, the removal of infected indwelling urinary catheters is one of the most effective methods used to resolve bacteriuria and CAUTIs[34,40]. However, removing the indwelling medical device, even combined with long course of antibiotics treatment[40], may not be sufficient for complete resolution of the infection, especially with the rise in antibiotic resistance observed in nosocomial settings[277]. In addition, implant removal upon infection in other device-associated infections like prosthetic valve endocarditis is not in itself efficacious[278-280], and thus may not be a suitable therapeutic approach in all instances. Understanding the pathogenesis of CAUTIs may lead to new and better ways to treat and prevent these diseases.

The optimized murine model of foreign-body associated UTI presented here is highly relevant for the investigation of the mechanisms underlying enterococcal-mediated CAUTIs since it couples biofilm production to enterococcal virulence during *E. faecalis* uropathogenesis. Findings from this study in this murine model led to the conclusion that the acute inflammatory response induced by urinary catheterization, partially responsive to glucocorticoid treatment, does not promote enterococcal infection rather the establishment of successful infections by this opportunistic pathogen results from the failure of the immune response, especially neutrophils, to prevent, block, or disrupt biofilm formation, rendering this process a major target in the fight against enterococcal CAUTIs (Fig. 31). This murine model represents a valuable and robust tool for understanding the mechanisms underlying urinary catheter-mediated cystitis and for the identification and characterization of novel biofilm determinants, bacterial virulence

factors as well as host responses pertinent to the pathogenesis of *E. faecalis* in the urinary tract. Importantly, it will serve as an ideal platform for testing anti-biofilm compounds and potential therapeutics for the prevention and treatment of enterococcal CAUTIs.

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CHAPTER THREE

CONSEQUENCES OF URINARY CATHETERIZATION ON UROPATHOGENIC ESCHERICHIA COLI PATHOGENESIS

Modified from: Guiton, P.S., *et al.* manuscript submitted.

ABSTRACT

Catheter-associated urinary tract infections (CAUTIs) caused by multidrug resistant pathogens including uropathogenic *Escherichia coli* (UPEC) constitute the majority of nosocomial UTIs and pose significant clinical challenges. Urinary catheterization elicits major histological and immunological alterations in the bladder that can favor microbial colonization and dissemination in the urinary tract. The present study reports the consequences of these biological perturbations on uropathogenic *Escherichia coli* (UPEC) pathogenesis and virulence-based preventative measures using an optimized murine model of foreign body-associated urinary tract infection (UTI). The findings obtained demonstrate that type 1 pili mediate UPEC adherence and invasion into bladder epithelial cells in catheterized bladders as seen in non-catheterized bladders as well as to the urinary implant. Specific targeting of type 1 pili with small molecule inhibitors in combination with trimethoprim/sulfamethoxazole prevents UPEC CAUTI. This study further demonstrates that bacterial reservoirs established during previous UPEC infection can serve as a nidus for urinary catheter colonization. This study provides novel insight into UPEC pathogenesis in the context of urinary catheterization, and investigates novel therapies that target critical mechanisms for this pathophysiology. By doing so, it establishes a proof-of-principle for the development of novel therapies to prevent and eventually treat these infections in the face of the rise of antibiotic resistant uropathogens.

INTRODUCTION

CAUTIs often arise from multidrug resistant Gram-positive and Gram-negative bacterial colonization and biofilm aggregation on the surface of indwelling urologic devices such as urinary catheters, rendering treatment very difficult[33,40,56]. Uropathogenic *Escherichia coli* (UPEC), the primary cause of community-acquired UTI, account for 50% of nosocomial UTIs, including CAUTIs[55]. Yet, very little is known about its pathogenesis following urinary catheterization. Urinary catheterization results in the disruption of the normal mechanical and antimicrobial defenses of the bladder[25,26,29,30,281]. Previous reports using human biopsies and rodent models of infections have shown that the catheterized bladder is edematous and highly inflamed with immune cell infiltration and pro-inflammatory cytokine production, an environment quite different from that which UPEC encounters in a non-catheterized bladder[62,71-73,75,77,282] . We hypothesized that these profound catheter-related changes may affect UPEC pathogenesis.

The UPEC pathogenic cascade has been extensively characterized in a non-catheterized murine model of cystitis. UPEC elaborate on their surface adhesive type 1 pili, which mediate binding to and invasion of superficial umbrella cells lining the bladder epithelium[60]. Once intracellular UPEC can escape into the cytoplasm, replicate rapidly and undergo morphological differentiation within bladder epithelial cells to produce mature intracellular bacterial communities (IBCs) of ~10^4-10^5 bacteria with biofilm-like properties[73,74]. UPEC then flux out from infected cells and can reinvade neighboring cells and start the process *de novo*[73]. This acute phase of UPEC infection can lead to the development of chronic cystitis, pyelonephritis, and the formation of quiescent intracellular reservoirs (QIRs) with absence of bacteriuria[77]. Detailed understanding of

the critical steps of this pathogenic cascade has led to the development of small molecule inhibitors called mannosides[84]. Mannosides specifically target the bacterial type 1 pili tip adhesin, FimH, which binds to mannosylated residues present on the surface of the bladder epithelium. Rationally designed to interfere and prevent FimH interaction with these residues, mannosides inhibit UPEC binding and invasion of the superficial umbrella cells during urinary tract infections (UTIs)[84]. Mannosides, in combination with existing antibiotic-based UTI therapy, have recently been shown to be effective in preventing and treating UPEC infections in non-catheterized infection models[87]. The present study investigates whether this therapeutic approach could be beneficial in the prevention and treatment of CAUTIs.

In this report, the optimized murine model of foreign body-associated UTI that closely mimics CAUTI[29] was used to investigate the consequences of urinary catheterization on the pathophysiology of UPEC infection. For these studies, several UPEC virulence parameters, including the contribution of type 1 pili, IBC formation, and QIR reactivation, were assessed. The results obtained indicate that urinary catheterization provides UPEC with the opportunity to exploit the extracellular milieu of the bladder via type 1 pili-mediated biofilm formation on the surface of the foreign body, which results in a shift in the niche population. Administration of mannosides in combination with trimethoprim/sulfamethoxazole prior to urinary catheterization prevents UPEC colonization of the urinary tract. This report provides important insights into the mechanisms underlying UPEC-mediated CAUTI, and informs efforts to design better therapeutic approaches to prevent and potentially treat these infections.

RESULTS

UPEC adherence, invasion, and IBC morphology are unaltered in catheterized bladders.

To assess the effects of urinary catheterization on UPEC colonization patterns in the urinary tract, 4-5mm platinum-cured silicone tubing sections (referred to as implants) were implanted in the bladders of C57Bl/6Ncr female mice, which were then immediately infected with $1\text{-}2x10^7$ CFU of the well-studied virulent UPEC strain UTI89[282] by transurethral catheterization. CFU enumeration was compared to non-implanted animals similarly infected with UTI89 up to 7 days post infection. UPEC colonized the bladder and kidneys of implanted animals to similar levels as in non-implanted animals (Fig. 32). In addition to the organs, UPEC was also recovered at very high titers from the implants, on which they established biofilms as determined by scanning electron microscopy at 48hpi (data not shown).

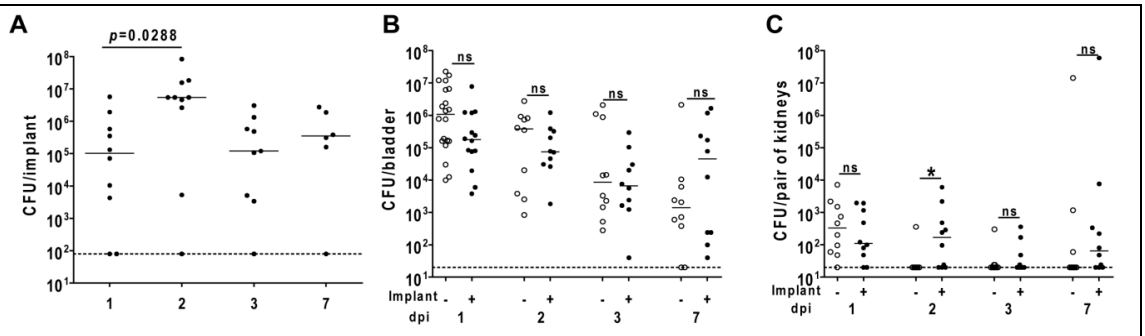

Figure 31: Bladder implantation does not alter the overall colonization pattern of UPEC. Graphs represent bacterial titers in log scale from retrieved implants **(A)**, homogenized bladders **(B),** and kidneys **(C)** from non-implanted (○) or implanted (●) animals infected with 1-2x10^7 CFU in 50µl inoculum of *E. coli* strain UTI89 for 24, 48, 72 hpi, or 7 dpi. Horizontal dashed lines represent the limit of detection for viable bacteria. Each symbol represents a mouse from two independent experiments with n=5/condition. The horizontal bars represent the median of each dataset; **p*<0.05, ***p*<0.005, and ****p*<0.0005 by the Mann Whitney U test.

IBC formation occurs in the pathogenesis of UPEC in non-catheterized patients[78] and has been shown in mouse models to be critical for infection. To assess the effects of urinary catheterization on IBC formation, implanted and non-implanted mice were infected with UTI89 as described above. *Ex vivo* gentamicin protection assays[62] performed at 3hpi revealed no statistical difference in either the extracellular or intracellular UPEC populations in the presence or absence of implants (Fig. 33), indicating no gross defect in bacterial invasion in implanted animals. IBC formation within both implanted and non-implanted bladders was assessed by LacZ staining and confocal scanning laser microscopy (CSLM) at 6hpi (Fig. 34A-C). Inoculation of UPEC into implanted animals resulted in significantly fewer IBCs with a median of 8 IBCs/bladder (*p*=0.0044) (Fig. 34B) compared to non-implanted animals in which IBC numbers ranged up to >250 IBCs/bladder with a median of 55 IBCs/bladder. Yet, bacterial CFU in implanted

bladders were similar to those in non-implanted animals (data not shown). This observation led to the reasoning that IBC morphology might be different in implanted animals. However, IBCs formed in implanted animals were observed to be overall similar in size and shape as those produced in non-implanted bladders (Fig. 34C). UPEC were also seen to produce multiple IBCs within one umbrella cell and filamentous bacterial clumps in both catheter implanted and non-implanted bladders at 6hpi (Fig. 35). UPEC colonization at 6hpi was selectively localized in the remaining umbrella cells and not observed in the exposed underlying epithelium in implanted bladders (Fig. 35). Together, these findings indicate that urinary catheterization negatively impacts IBC formation by UPEC, possibly due to a correlated increase in exfoliation of the superficial umbrella cells[283].

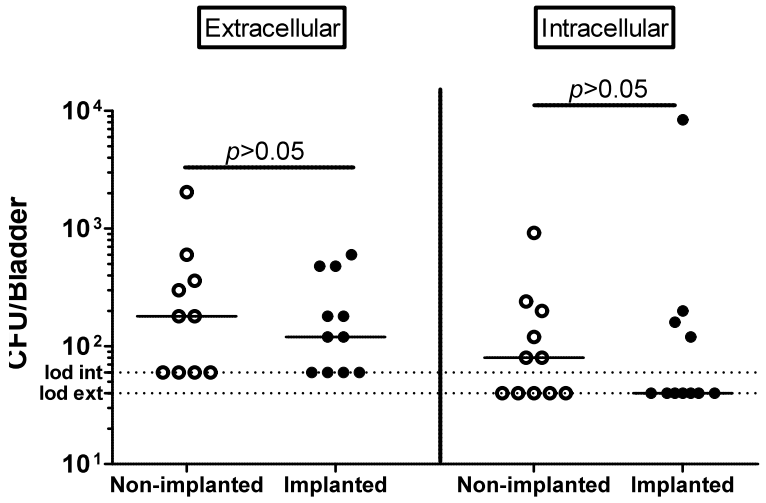

Figure 32: No defect in UTI89 invasion following implantation.

Graph represents bacterial titers from homogenized bladders from non-implanted (○) or implanted (●) animals infected with UTI89 3hpi following *ex vivo* gentamicin protection assay. Horizontal dashed lines represent the limit of detection for viable bacteria (Int=intracellular, Ext=Extracellular). Each symbol represents a mouse from two independent experiments with n=5/condition. The horizontal bars represent the median of each dataset; *p* value by the Mann Whitney U test.

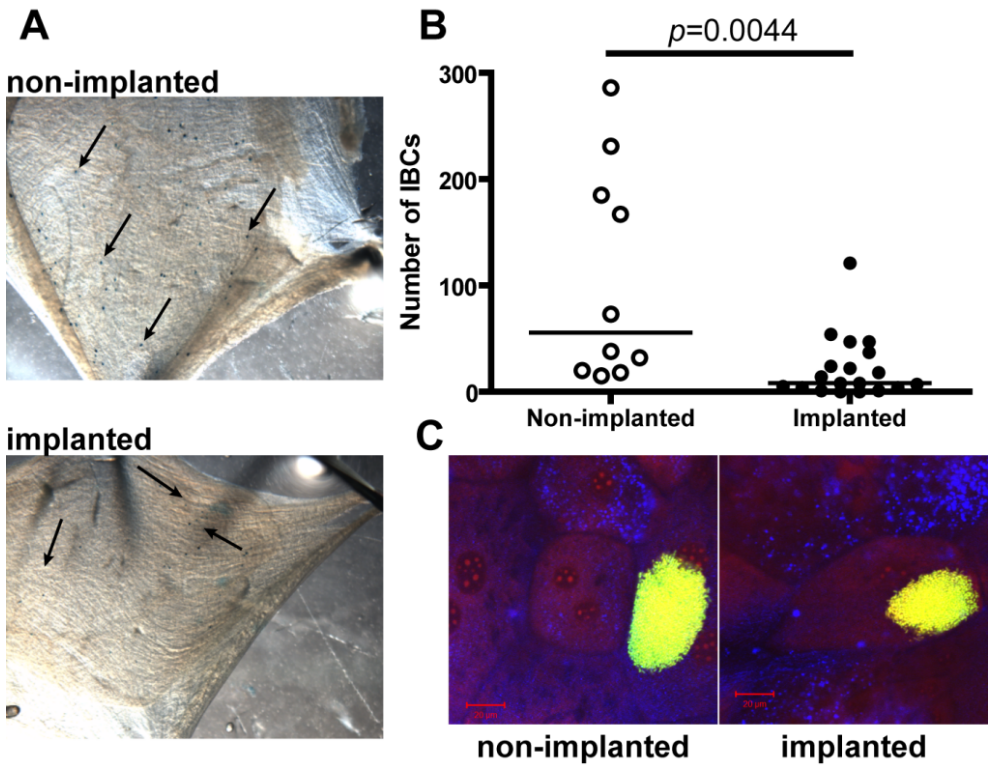

Figure 33: Uropathogenic *E. coli* produces IBCs in the superficial umbrella cells of implanted bladders.

(A) Representative images of splayed bladders of female C57Bl/6Ncr mice infected with UTI89 6hpi in the absence (non-implanted) or presence (Implanted) of implants following LacZ staining. Each black arrow indicates a purple speck, indicative of an IBC. **(B)** Quantification of IBC formation following LacZ staining at 6hpi. Each symbol represents IBC number from a single mouse from two independent experiments with n=5/group. *p* value obtained from the Mann Whitney U test. **(C)** Representative CLSM images of whole bladders from non-implanted and implanted animals infected with UTI89 ectopically expressing GFP (Green), stained with DNA dye SYTO83 (Red) and Alexa-fluor 633-conjugate of WGA (Blue) reveal the presence of IBC within umbrella cells. Scale bar =20µm.

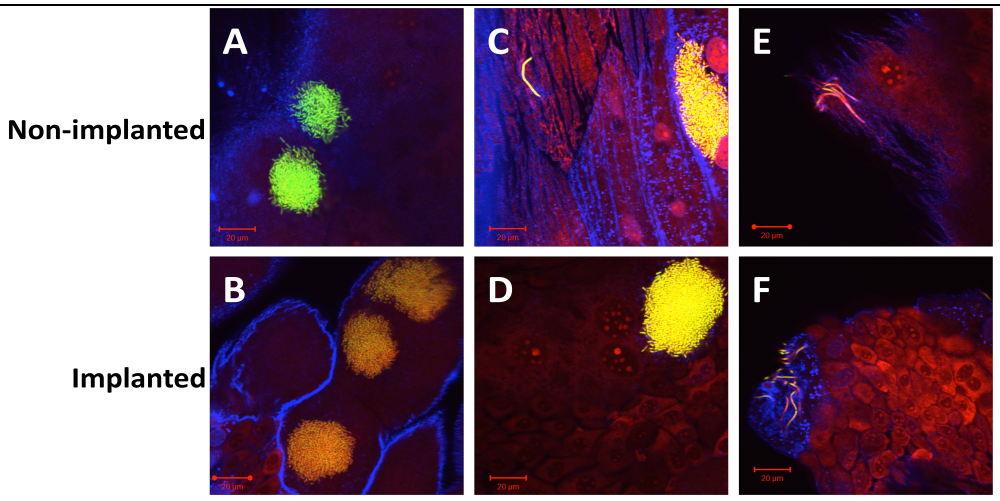

Figure 34: IBC and filamentation occur following urinary catheterization

Representative CLSM images of whole bladders from non-implanted and implanted animals infected with UTI89 ectopically expressing GFP (Green), stained with DNA dye SYTO83 (Red) and Alexa-fluor 633-conjugate of WGA (Blue) reveal the presence of multiple IBCs within single umbrella cells **(A-B)**, that unlike non-implanted bladders **(C),** the underlying epithelium is exposed following urinary catheterization **(D-F)**, depict the absence of bacterial colonization of the exposed underlying epithelium in implanted animals **(D-F)**, and the presence of filamenting bacteria in umbrella cells **(E-F)**. Scale bar =20µm.

Bacteria originating from bacterial reservoirs can seed urinary implant colonization.

One troubling possible outcome of the UPEC pathogenic cascade is the establishment of quiescent intracellular reservoirs (QIRs) in the underlying epithelial layers, which have been shown, can be a source of recurrent UTIs (rUTIs)[77,284]. QIRs were shown to be reactivated following treatment with protamine sulfate, a chemical that leads to exfoliation of the superficial umbrella cells of the uroepithelium [77]. Like protamine sulfate, urinary catheterization causes severe damage to the protective

uroepithelial layer[77]. Thus, urinary catheterization might also reactivate QIRs, resulting in bacteriuria, catheter colonization and further dissemination. To test this hypothesis, mice were infected with 1-2x10^7 CFU UTI89HK::GFP and infection allowed to resolve over the course of 2 weeks. On day 14 post-infection, urine was collected from each animal to assess infection state prior to urinary implantation of a subset of these animals. Those animals in which titering of urines 14 dpi indicated bacteriuria ($\geq 10^4$ CFU/ml) were considered to have active (non-resolved and/or recurrent) infection and were removed from further analysis. The remaining mice were presumed to either have completely cleared the infection or to have established QIRs. 3 or 5 days post implantation, reservoirs activation was assessed by bacterial colonization of implants and bladder. On day 3 post implantation, UPEC UTI89HK::GFP was recovered from implants in 3 of 26 implanted mice (~11.5%) and one mouse had bladder colonization (> 10^4 CFU) compared to none of 22 similarly infected but non-implanted animals (Fig. 36A). For mice assessed at 5 days post-implantation, UTI89HK::GFP was recovered from implants of 4 out 32 animals (~13%) with two of them having bladder titers greater than 10^4 CFU/ml (Fig. 36B) compared to 0 out of 23 in non-implanted animals. Interestingly, there were overall significantly fewer bacteria recovered from the bladders of implanted animals compared to non-implanted animals at 5 dpi (p=0.017; Fig. 37B), suggesting that either reactivated reservoirs are cleared by the immune response prior to day 5 following implantation or increased exfoliation prevents establishment of persistent infections. Together, these data indicate that urinary catheter colonization can occur from previous urinary infections even if those infections appear by bacteriuria counts to have been resolved.

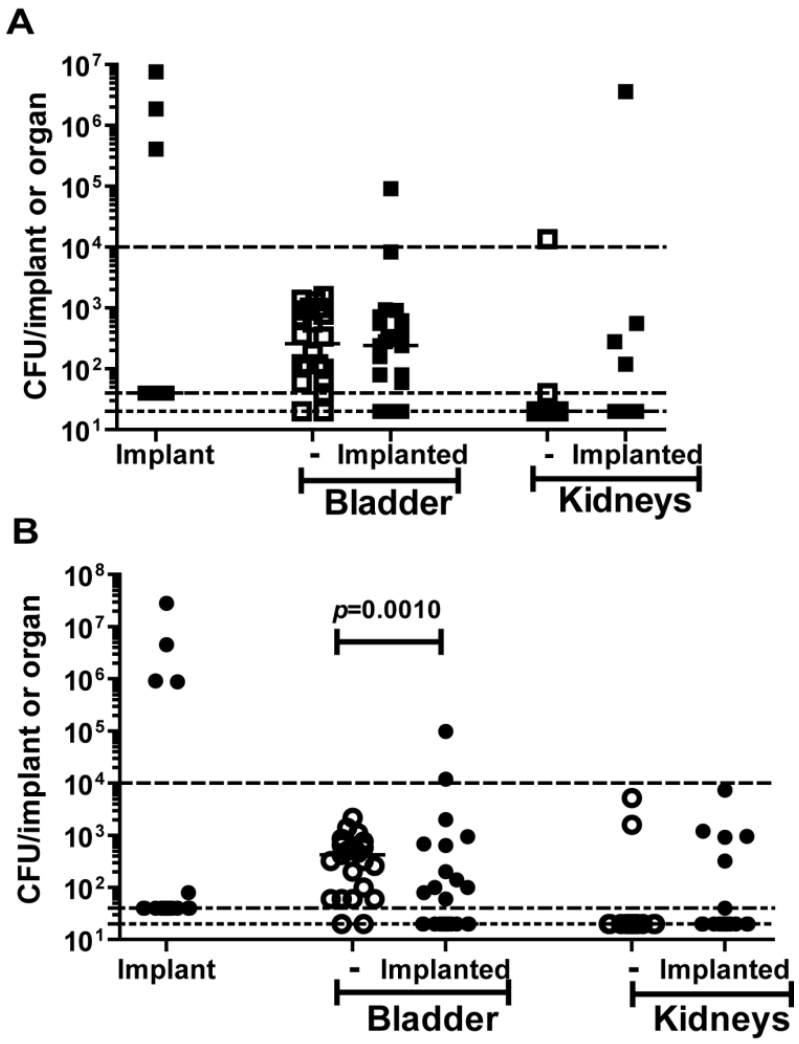

Figure 35: Bacterial reservoir reactivation can lead to implant and bladder colonization.

Graphs represent bacterial titers in log scale recovered from implants, homogenized bladders and kidneys of non-bacteriuric animals 14days post infection with UTI89HK::GFP that were non-implanted or implanted for 3 day **(A)** or 5days **(B)**. Horizontal dashed lines represent the limit of detection for viable bacteria. Each symbol represents a mouse from at least two independent experiments with n=10-20/group/experiment. The horizontal bars represent the median of each dataset; *p* value by the Mann Whitney U test.

FimH is required for biofilm formation and UPEC colonization of the urinary tract following catheter implantation.

Biofilm formation is a critical component of UPEC CAUTI pathophysiology[33,37]. Type 1 pili are major UPEC virulence factors that have been shown to be critical for biofilm aggregation, IBC formation, and other aspects of UPEC uropathogenesis. Thus, the contribution of these extracellular pili as well as other UPEC fibers, including curli which contribute to biofilm formation[285] or S pili associated with *E. coli* clinical isolates producing strong biofilms[286-288], to biofilm formation in filtered human urine under flow conditions and UPEC-mediated CAUTI *in vivo*. Deletion of the gene for the tip adhesin of type 1 pili, *fimH*, in UTI89 resulted in significantly ($p<0.0001$) lower biomass (Fig. 37A) and an approximate 2-fold reduction in adherent viable bacteria (Fig. 37B) in biofilms formed in human urine *in vitro*. These data indicate that type 1 pili are a major contributor to UPEC biofilm formation in urine. The biofilm defect was specifically associated with the *fimH* mutant under these conditions, and was not observed following deletions of the *sfa* operon to prevent S pili formation, *csgA* required for curli fiber formation, or a component of the flagellar system *fliC* (data not shown).

In vivo, similar to findings in a murine model of cystitis, UTI89Δ*fimH* is severely attenuated in the murine model of foreign body-associated UTI (Fig. 37C). UTI89Δ*fimH* displayed >3 log fewer CFU in the bladder and was unable to ascend to the kidneys at 24hpi. Further, deletion of *fimH* resulted in significant reduction in implant colonization ($p<0.0001$).

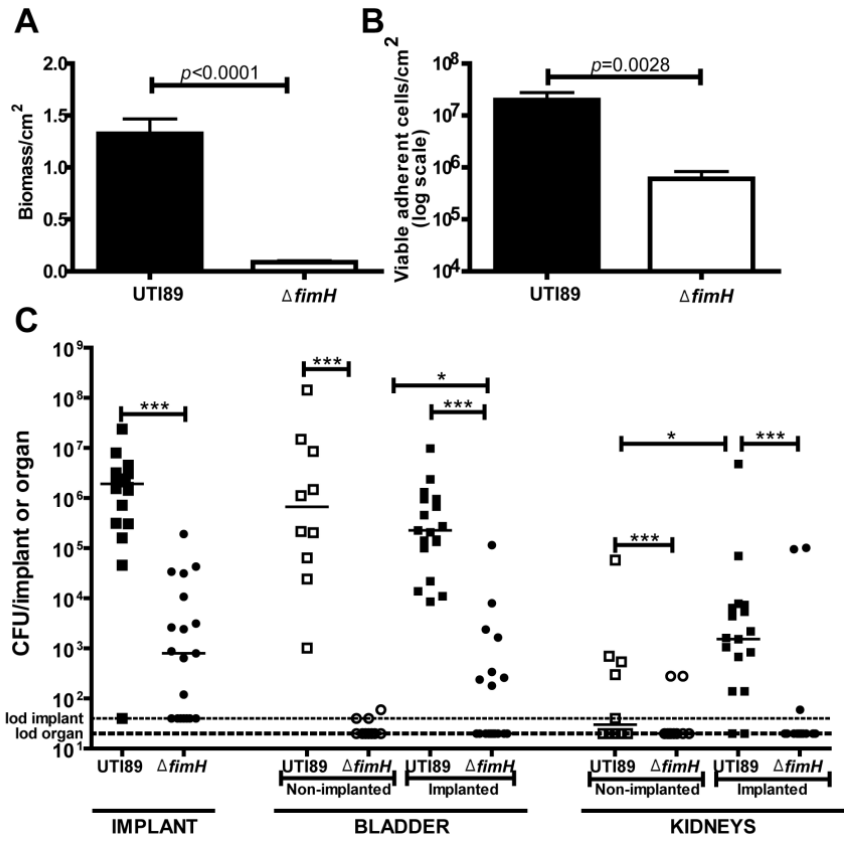

Figure 36: Deletion of *fimH* reduces biofilm formation and attenuates UPEC virulence.

Graphs represent crystal violet based quantification (A) and CFU enumeration in logarithmic scale (log scale) (B) of 24h old UTI89 and UTI89Δ*fimH* (Δ*fimH*) biofilms under human urine flow on silicone tubing at 37ºC indicating that Δ*fimH* is defective in biofilm formation under these conditions. Bars represent mean of three independent experiments, error bars indicate standard error of the mean (SEM). *p* values from Mann Whitney U test. (C) Graph represents bacterial titers in log scale recovered from implants, homogenized bladders and kidneys of non-implanted (open symbols) and implanted (closed symbols) infected with either UTI89 (square) or Δ*fimH* (circle) for 24h. Horizontal dashed lines represent the limit of detection for viable bacteria. Each symbol represents a mouse from at least two independent experiments with n=5/group. The horizontal bars represent the median of each dataset; **p*<0.05 and ****p*<0.0005 by the Mann Whitney U test.

Similar to *in vitro* experiments, S-pili are not required for CAUTI since UTI89Δ*sfaA-H* is as virulent as wild type UTI89 and a double deletion of both *sfaA-H* and *fimB-H* recapitulated the UTI89Δ*fimH* phenotype (Fig. 38A). Furthermore, components of the curli system important for biofilm formation *in vitro* under certain conditions, but not in human urine (Fig. 3A-B), were also dispensable during CAUTI (Fig. 38B). The residual binding to implants and bladders in implanted animals could therefore be attributed to other pili or biofilm determinants. Together, these data strongly suggest that the tip adhesin FimH of type I pili is a critical determinant of UPEC virulence in mediating biofilm formation and virulence during CAUTI.

Mannoside treatment reduces IBC formation.

Having established that FimH is required for UPEC virulence in implanted bladders, this adhesion was investigated as a potential therapeutic target for CAUTI using small molecules inhibitors designed to interfere with FimH binding to mannosylated residues[84]. This family of small molecules, called mannosides, has recently been shown to prevent acute and chronic UPEC infections and potentiated the effectiveness of antibiotics in combinatorial treatment[87]. To investigate the potential therapeutic effects of mannosides on CAUTI, the inhibitory effects of methyl-α-D-mannopyranoside (methyl mannose) on UTI89 biofilm formation in urine under flow was determined. Similar to the deletion of *fimH* (Fig. 37A-B), UTI89 biofilms grown in presence of 1% methyl mannose had significantly reduced biomass (p=0.0022) and biofilm-adherent cells (p=0.0012), compared to untreated controls (Fig. 39). Since methyl mannose is a FimH antagonist, these data confirm the critical role of type 1 pili to biofilm formation in urine as was previously described for biofilms formed in LB media.

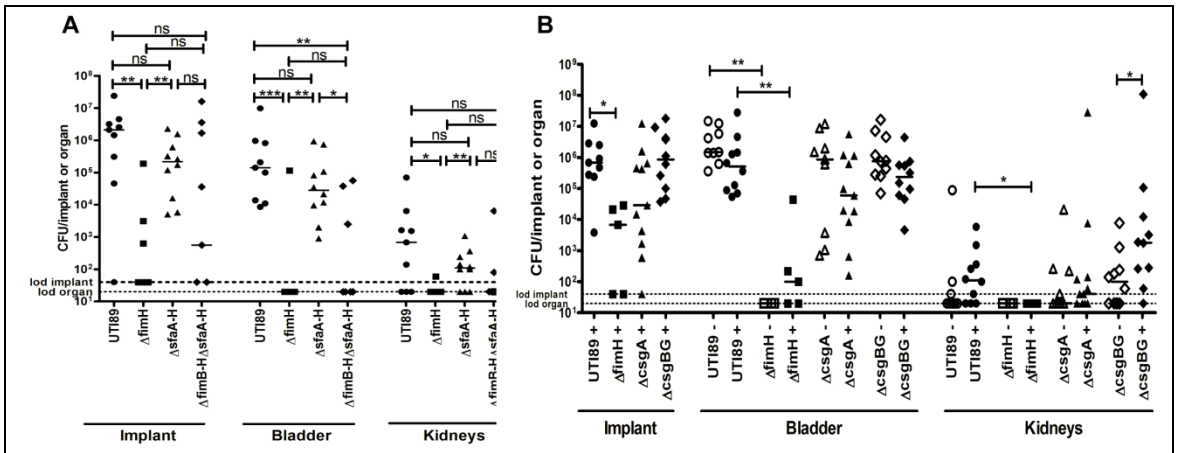

Figure 37: S pili and curli are not critical for UPEC virulence following urinary catheterization.

Graphs represent bacterial titers in log scale recovered at 24hpi from implants, homogenized bladders and kidneys of **(A)** implanted animals infected with either UTI89 (●) or UTI89 mutant strains deficient in type 1 pili, Δ*fimH* (■), S pili Δ*sfaA-H*(▲), both type 1 and S pili Δ*sfaA-H*Δ*fimB-H*(♦) and **(B)** non-implanted (open symbols) or implanted (closed symbols) animals infected with UTI89 (○,●), Δ*fimH* (□, ■) or UTI89 mutant strains deficient in curli components Δ*csgA* (Δ,▲) and Δ*csgB*Δ*csgG* (♦,◊). Horizontal dashed lines represent the limit of detection for viable bacteria. Each symbol represents a mouse from at least two independent experiments with n=5/group. The horizontal bars represent the median of each dataset; *p<0.05 and ***p<0.005 **p<0.0005 by the Mann Whitney U test.

The effects of mannoside treatment were then assessed *in vivo* by using IBC formation as well as implant and urinary tract colonization as benchmarks of disease progression. Mice were treated intraperitoneally (i.p.) with saline or 5mg/kg of mannoside ZH56[84] in PBS 30min prior to urinary implantation. Mannosides, including ZH56, are more potent than methyl mannose in preventing and disrupting UTI89 biofilm *in vitro*. Methyl mannose, previously shown to inhibit UTI89 biofilm at 100mM in LB

using the 96-well biofilm assay[289] was used for the *in vitro* urine catheter biofilm studies while the more potent mannoside was reserved for the *in vivo* studies due to its increased affinity and stability. Catheter implantation was immediately followed by transurethral inoculation of UTI89. IBC formation and bacterial colonization were assayed by LacZ staining and CFU enumeration of implants, bladders, and kidneys at 6hpi and 24hpi, respectively. As shown in Fig. 40A and 40B, mannoside treatment further reduced IBC formation (p=0.0051) and bladder colonization (p=0.0114) in implanted animals at 6hpi, suggesting that this treatment prevents intracellular infection. While eliminated from their intracellular niche, data further indicated that UPEC were able to persist in the extracellular milieu where they can colonize the surface of the implants to relatively similar levels as saline-treated animals (p=0.0547) (Fig. 40B). No statistical difference was observed in kidney colonization in the presence or absence of mannosides (Fig. 40B). By 24hpi, a time point at which the mannosides have been eliminated from the bladder[87], similar bacterial loads were recovered from implants, bladders, and kidneys in implanted animals in the presence or absence of mannoside treatment (data not shown). Furthermore, treatment with mannosides did not have an effect on established UPEC CAUTI (data not shown).

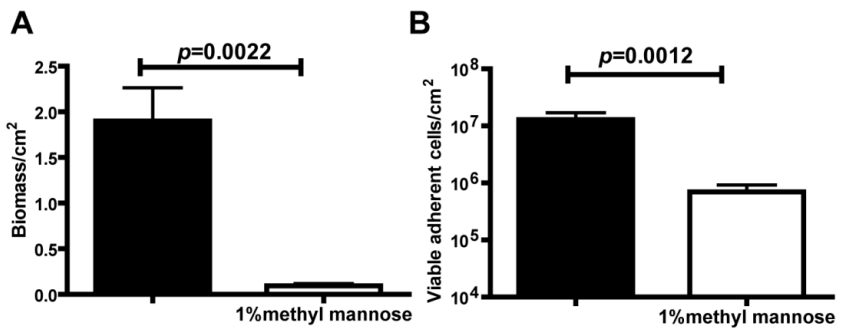

Figure 38: Methyl mannose inhibits UPEC biofilm in human urine

Graphs represent crystal violet based quantification **(A)** and CFU enumeration in logarithmic scale (log scale) **(B)** of 24h old UTI89 biofilms in human urine with or without 1% methyl mannose under flow on silicone tubing at 37ºC indicating that methyl mannose prevents UPEC biofilm formation. Bars represent mean of three independent experiments, error bars indicate standard error of the mean (SEM). *p* values from Mann Whitney U test.

Mannoside treatment increases the efficiency of TMP-SMZ in preventing UPEC colonization.

In order to examine whether mannosides could prevent establishment of CAUTI when used in combination with antibiotics, animals were treated with 54 and 270µg/ml of trimethoprim-sulfamethoxazole (TMP-SMZ), respectively, in their drinking water for 3 days and then treated with saline or mannoside (5mg/kg) i.p. 30 min prior to implantation and bacterial inoculation. At 6hpi, UPEC colonized the implants and bladders at significantly lower levels in animals that only received antibiotics compared to those who received water or were only administered mannoside (Fig. 40B). Interestingly, mannoside treatment in addition to TMP-SMZ further decreased UPEC colonization of implants, bladders, and kidneys compared to treatment with antibiotic alone ($p<0.0005$ in all

cases). Together, these findings indicate that virulence-targeted therapies in combination with established antibiotic treatment can help prevent or delay the onset of CAUTI.

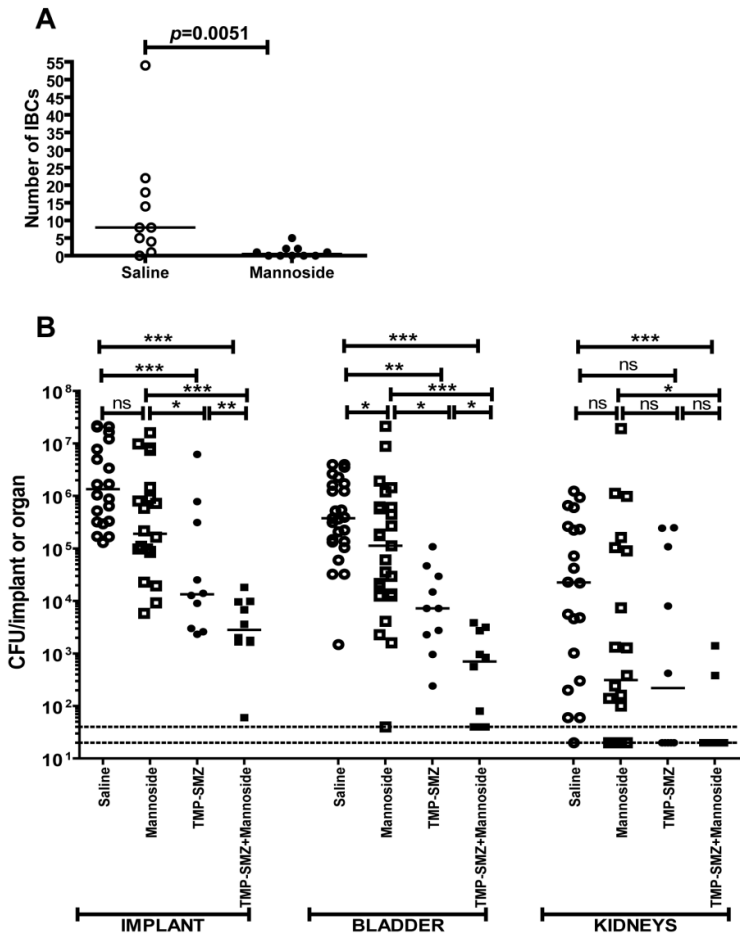

Figure 39: Mannoside treatment prevents IBC formation and UPEC virulence when use in combination with TMP-SMZ. (A) Graph represents IBC enumeration from LacZ staining of splayed bladders of female C57Bl/6Ncr mice treated i.p. with mannoside or saline prior to transurethral implantation and inoculation with UTI89 6hpi. **(B)** Graph represents bacterial titers in log scale recovered at 6hpi from implants, homogenized bladders and kidneys of animals treated with saline (○), mannoside (□),TMP-SMZ (●) and TMP-SMZ+Mannoside (■) prior to urinary implantation and inoculation with UTI89. Horizontal dashed lines represent the limit of detection for viable bacteria. Each symbol represents IBC number or bacterial titer from a single mouse from two independent experiments with n=5/group. The horizontal bars represent the median of each dataset; *p<0.05, **p<0.0005, ***p<0.0005, ns corresponds to p>0.05 by the Mann Whitney U test.

DISCUSSION

UPEC is the major etiological agents of CAUTI[56]. Yet, the molecular mechanisms of urinary catheter and bladder colonization following urinary catheterization have not been elucidated. Studies in an optimized murine model of foreign body-associated UTI[281] show that urinary catheterization favors UPEC exploitation of the bladder extracellular milieu. This occurs via type 1 pili-dependent biofilm formation on the surface of silicone implants in the murine bladder. The data further indicate that of the biofilm determinants tested, type 1 pili are necessary for implant, bladder, and kidney colonization during CAUTI; providing definitive experimental evidence for previous reports postulating that type 1 pili may be required for UPEC persistence during CAUTIs[290]. Interestingly, *fimH*-deficient UPEC strains have the ability to adhere to some extent to the surface of the foreign body, probably using other biofilm determinants such as other chaperone-usher pili systems, curli, or surface adhesins[43].

In addition to colonizing the foreign body in the bladder lumen, UPEC is able to exploit intracellular niches in implanted animals, albeit to a lesser degree than in non-implanted animals, by invading and producing IBCs in the early stages of infection. Reduced IBC formation in implanted animals may be a result of loss of the host superficial facet cells in which IBCs form due to increased exfoliation or damage to the uroepithelium following urinary catheterization[25,26,29,30,281,283,291]. Nonetheless, this finding is of particular interest for treatment strategies against UPEC-mediated CAUTI. In humans, removal of the contaminated urinary catheter is the preferred method for treatment of these infections[34,40]; however, the presence of bacteria in an intracellular compartment protected from host immune defenses and antibiotic treatment requires

more comprehensive approaches as intracellular UPEC can lead to re-infection of a novel catheter or serve as a nidus for future UTI.

Quiescent intracellular reservoirs are an important outcome of UPEC pathogenic cascade because they are proposed to be a mechanism of recurrent UTI following damage to the uroepithelium[77]. Findings from the current study indicate that urinary implantation of animals with a history of UTI can lead to bladder infection and implant colonization with the UPEC causing the first infection. This finding suggests that in addition to introduction of extracellular and periurethral bacteria [36], urinary catheter colonization can occur from bacteria originating from pre-existing reservoirs or other niches within the urinary tract not appreciated by assessment of bacteriuria. Interestingly, by day 5 following implantation in animals with a history of UTI, there is overall a significant reduction in the number of QIRs compared to that in non-implanted animals as assessed by bladder CFU. This reduction in bacterial load could be a result of enhanced immune-mediated clearance of infected cells or to exfoliation of infected cells in implanted animals. These hypotheses are currently being evaluated.

The identification of FimH as a critical virulence factor during UPEC CAUTI provides an interesting avenue for the development of novel preventative measures against these infections. The results presented here suggest that the use of small molecules inhibitors, such as mannosides, in combination with existing UTI treatment regimes, can lead to prevention or delay of UPEC colonization. Mannosides, were rationally designed to interfere with FimH-mediated binding to mannosylated proteins on the surface of the uroepithelium[84]. Recent studies show that the use of mannosides in combination with antibiotics is highly effective in preventing IBC formation and acute

stages of UPEC infection as well as treating chronic cystitis[87]. Similarly, pretreatment with mannosides further prevents IBC formation following UPEC infection of implanted bladders and reduces UPEC binding to the uroepithelium. However, the presence of the abiotic implant surface provided a favorable environment for adherence of extracellular bacteria. The inability of mannoside treatment to eliminate UPEC from the implant, given that the experimental evidence that mannose inhibits UPEC biofilm on catheter material in urine *in vitro*, may be due to the lack of urine flow in this implanted murine model. It is possible that if it were possible to truly catheterize mice in a manner analogous to clinical catheterization of humans in which urine flows through the catheter that mannoside may have a more efficacious effect on implant clearance. Nonetheless, by preventing invasion and shifting UPEC niche to the extracellular milieu, mannoside enhanced the bacteriocidal efficacy of antibiotics, such as TMP-SMZ, which does not cross host cell membranes. Mannosides could be used in combination with bacterial interference strategy being offered as alternatives in the prevention of CAUTI. Previous reports have shown that pre-colonization of urinary catheters with an avirulent *E. coli* strain 83972 delays the onset of CAUTI in catheterized patients[292]. Thus, it is quite possible that if used in combination with mannosides, UPEC will be kept from the intracellular niche as well as from binding to the catheter during bacterial interference with the avirulent strain. Though, the avirulent strain should be able to colonize the catheter in a type 1-independent manner, which is not the case for *E. coli* strain 83972[293]. The therapeutic effects of mannoside could not be recapitulated in our model of CAUTI (data not shown), possibly because the small molecules are ineffective at disrupting established biofilms *in vivo*. It is a well-established fact that the extracellular matrix of

bacterial biofilms is impermeable to antimicrobial and antibiotics, providing a safe haven for the microbes within[42,54].

Urinary catheterization is a necessary medical procedure that causes major damage to the urinary tract. Pathogens, such as UPEC, take advantage of this compromised environment to exploit new and existing niches and establish severe infections. This report uncovers important molecular mechanisms underlying UPEC pathogenesis following urinary catheterization. It raises important questions regarding the deleterious consequences of urinary catheterization and the origins of urinary catheter colonization. These novel aspects of CAUTI pathophysiology should thus be taken into consideration for the development of anti-virulence based preventative and therapeutic approaches against these infections.

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DISCUSSION AND FUTURE DIRECTIONS

The management of CAUTIs has recently gained considerable interest in healthcare settings[20,21,56] due to the rise in multidrug resistant pathogens and given the significant medical and societal burdens associated with these infections[12,56]. However, the paucity of data describing the molecular mechanisms underlying the pathophysiology of CAUTIs contributes to the high prevalence, difficult diagnoses, and ineffective treatment regimens associated with these infections[5,12].

In the present dissertation, *in vitro* biofilm assays and a murine model were optimized to closely replicate the key characteristics of CAUTIs. These served as valuable tools to unravel previously unappreciated details underlying the detrimental effects of urinary catheterization on bladder homeostasis and revealed the microbial and immunological factors contributing to enterococcal and UPEC establishment of CAUTIs. Surprisingly, some *in vitro* biofilm determinants, such as Atn, GelE, and UPEC S pili were not found to play a role in biofilm formation based on the CAUTI model whereas enterococcal sortases and UPEC type 1 pili were major contributors of virulence. Furthermore, studies revealed that the pathogenic cascade of UPEC is quantitatively altered in presence of foreign bodies due to a multitude of factors in addition to urothelial exfoliation[5,12]. Importantly, evidence presented in this dissertation show for the first time that bacteria reservoirs in the bladder established during previous infections are a potential third source for urinary catheter colonization. This is a risk that has not been previously considered and has major implications for clinical management and decision-making in the context of CAUTIs and other medical-associated infections. Ultimately, this study yielded insight towards the development of novel therapeutic approaches by

establishing the efficiency of specific inhibitors of UPEC type 1 pili tip adhesin FimH, namely mannosides, as alternative virulence-based preventative measures in the fight against CAUTIs.

In addition to the milestones achieved in unraveling the molecular factors involved in the pathogenesis of *E. faecalis* and UPEC CAUTIs and unappreciated aspects of CAUTI pathophysiology, this dissertation has multiple ramifications and opens up exciting avenues of research in various areas ranging from basic science research to clinical management of CAUTIs and other biofilm-associated infections.

Implications for enterococcal and UPEC pathogenesis:

Findings from this dissertation represent advance our understanding in the study of *E. faecalis* uropathogenesis as well UPEC pathogenesis in the context of urinary catheterization. Biofilm formation on the surface of urinary catheters, a central component of CAUTIs, was demonstrated here to be an essential virulence property for *E. faecalis* colonization of and persistence within the urinary tract. *In vitro* studies performed in this work showed that the enterococcal autolytic factor Atn mediates DNA release at a specific time during the establishment of DNA-dependent *E. faecalis* biofilms. Extracellular DNA (eDNA), was found to be a major component of the extracellular matrix of enterococcal biofilms *in vitro*, and to be critical for the maturation and architectural stability of these structures. These findings are in agreement with previous reports by Thomas *et al*, which showed that gelatinase (GelE) contributes to DNA release during biofilm formation by regulating autolytic processes probably via cleavage of Atn[103,104]. GelE is a well-established virulence factor in a rabbit model enterococcal endocarditis where biofilm formation occurs on the surface of heart

valves[221]. Yet, Atn and GelE were dispensable for biofilm formation and virulence in the urinary tract in the CAUTI model. These findings question whether eDNA is important for biofilm formation in the urinary tract. Investigation of the regulation of autolytic processes in *E. faecalis* will help uncover complementary pathways involved in DNA release, as DNA-dependent biofilm formation may be critical in other enterococcal infections and in an agricultural environment[221,294]. Furthermore, *E. faecalis* may employ eDNA-independent pathways to establish biofilms on the surface of urinary catheters.

The enterococcal factors identified to promote biofilm formation and virulence in the urinary tract were sortases SrtA and SrtC, the *e*nhanced *e*xpression of pheromone *p*rotease (Eep), and the transcription factor AhrC. Thus, sortase substrates, which are cell surface proteins, may represent attractive targets for antimicrobial therapies given their ubiquitous nature and various functions in bacterial physiology and pathogenesis[116,143]. *E. faecalis* encodes over 30 LPxTG motif containing proteins[116], including the *e*ndocarditis and *b*iofilm-associated *p*ilus (Ebp), a structure which contributes to enterococcal UTI pathogenesis[107] (Nielsen, H.V., Guiton, P.S., *et al*, manuscript in preparation). Expression studies *in vitro* and *in vivo* and direct mutagenesis of candidate genes may help uncover relevant biofilm and urovirulence-promoting substrates. As for UPEC, of the known biofilm determinants examined, including the amyloid fibers curli and S pili, only type 1 pili were found to be essential for biofilm formation and virulence in the murine model of foreign body associated UTIs.

The physiological requirements to produce and maintain biofilms in the urinary tract require further research. Genetic and biochemical approaches can be used to uncover the molecular details of the interactions within biofilm communities. Microarray

analyses of cells extracted from *in vivo* biofilms at different time during the infection process can identify genes that are important for metabolic processes, extracellular matrix formation, or immune evasion during CAUTI. In addition to microarray analyses following infections, identification of SNPs between *E. faecalis* OG1RF and OG1X strains can provide potential candidates for novel biofilm determinants and virulence factors. In collaboration with Kimberly Kline, Ph.D., Fredrik Henriques and Brigitta Henriques-Normark, M.D., Ph.D., 69 SNPs were identified in coding regions between the two strains and 11 were sequenced and confirmed (Tables 1 and 2). These genes have putative functions ranging from cell surface proteins to energy transduction. Investigation of their roles to *E. faecalis* biofilm and virulence will provide more insights into the drastic defect observed with OG1X *in vivo.*

Furthermore, the composition of the extracellular matrix of both *E. faecalis* and UPEC biofilms can be determined by isolating the matrix of biofilms from the surface of urinary implants from mice and from urinary catheters isolated from human patients and analyzing them using and optimizing previously established methods[65]. The molecular basis of how these structures change overtime can be investigated to provide insight into the biofilm developmental processes. Ideally, the constituents of these extracellular matrices, if identified and characterized, can be useful targets for the disruption and dissolution of biofilms on the surface of urinary catheters.

Table 1: List of the putative functions of the 69 SNPs identified in coding regions between OG1RF and OG1X

Putative functions	Number of different genes	Putative functions	Number of different genes
Replication	1	Lipid transport and metabolism	1
Transcription	8	Transport, unknown substrates	4
Translation	3	Energy metabolism	4
Inorganic transport	1	Membrane/cell wall	10 with 2 putative SrtA substrates
Nucleoside transport and metabolism	3	Signal transduction	1
Amino acid transport and metabolism	8	Defense mechanism	4
Carbohydrate transport and metabolism	4	Unknown function and hypothetical proteins	17

Table 2: List of 11 genes with sequenced and confirmed SNPs between OG1RF and OG1X

Gene with confirmed SNPs	Putative function	AA Change OG1RF → OG1X
EF0243	Branched-chain amino acid transport system II carrier protein (bnrQ)	Val→ Iso
EF3157	Glycosyl hydrolase, family 65	Ser →Asn
EF1513	Pheromone binding protein	Ser →Asn
EF1218	Spermidine/putrescine ABC transporter, permease protein	Pro →Ser
EF2575	Carbamate kinase	Gly →Asp
EF2983	Glutamyl-tRNA(Gln) amidotransferase, A subunit, putative	Ala →Thr
EF3285	PTS system, IIC component	Ser →Phe
EF3290	Sensor histidine kinase	Glu →Lys
EF1778	Phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase (purH)	Arg →Gln
EF2587	Inosine-uridine preferring nucleoside hydrolase	Arg →Thr
EF1349	Glycosyl hydrolase, family 13	His →Tyr

Remarkably, the establishment of persistent enterococcal and UPEC CAUTIs occur despite the induction of an acute inflammation response following urinary catheterization. The host immune response within the catheterized murine bladder consists of the onset of massive bladder wall edema due to an increased in vascular permeability, the production of pro-inflammatory cytokines, and the recruitment of myeloid cells, especially neutrophils. This robust response fails to prevent and clear bacterial infection. One hypothesis is that the acute immune response to a urinary catheter alters the adequate response to uropathogens or favors biofilm formation as shown for Methicillin-Resistant *S. aureus*[295]. Unlike *E. faecalis*, UPEC induces a robust

inflammatory response in non-catheterized animals[76,190,239]. Comparison of this immune response to that elicited during UPEC-mediated CAUTI can provide insight into the above hypothesis. Alternatively, *E. faecalis* and UPEC may employ several molecular immune evasion mechanisms to withstand and escape the deleterious effects of the immune system activated during CAUTI, such as subversion of neutrophil killing, invasion into the host cells[260,261,266,269,296-298], or incorporation of host-derived components to produce biofilms as shown for Nontypeable *Haemophilus influenza*[157,299], thus potentially rendering the biofilms less immunogenic.

This dissertation focused on the immune response 24h following urinary implantation with or without bacterial challenge. It will be informative to examine the changes of the immune response over time. This can be achieved by microarray and quantitative real time PCR analyses of bladder cells at different times following urinary implantation in the presence or absence of the bacteria of interest. Not only will these experiments allow the identification of the immune pathways leading to bladder inflammation after catheterization, they will help uncover the effects of bacterial colonization on these immune pathways. A pathway that is activated in response to bacteria and detrimental to the infectious agent can be modified and exploited for enhancing the antibacterial response whereas infection-promoting pathways could be targeted for downregulation or inhibition.

It will be of great interest to investigate the mechanisms underlying UPEC and enterococcal dissemination to the kidneys as well as the complications ensuing from urinary catheterization, including pyelonephritis and bacteremia. The onset of edema may cause reflux to the kidneys[300], and hence may promote bacterial colonization of these

organs. On the other hand, bacteria may have mechanisms to hijack the host cells and disseminate to other organs. The role of the host in bacterial dissemination can potentially be addressed following experimental depletion of host immune populations of interest.

Implications for microbial physiology and intra- and inter-microbial interactions:

CAUTIs are polymicrobial in nature[37,57,58]. Thus, these studies should be extended to mixed microbial communities given that the molecular interactions within constituents of polymicrobial communities on the surface of urinary catheters are poorly defined. Are all microbes found together or in specific consortia? Do they interact with each other to the same extent? Is there particular microbial associations during CAUTI progression as suggested by Macleod *et al.*[58]? What are the molecular factors underlying these preferential associations? Is the host immune response altered in presence of various bacteria? Is the host response to one pathogen favorable or detrimental to another? Introduction of other uropathogens such as *P. aeruginosa, P. mirabilis*, and other Gram-positive bacteria while studying CAUTI will contribute to a better understanding of the intra and inter-species interactions occurring during these infections.

It is proposed that *E. coli* and *Enterococci* are among the first to colonize urinary catheters and the microbial population is altered over time with colonization by *P. aeruginosa*, *P. mirabilis*, *Providencia stuartii*, *Morganella morganii*[37]. Data obtained in the course of this thesis indicate that live *E. coli* UTI89 and supernatants from stationary *E. coli* cultures inhibit enterococcal biofilm *in vitro* (Fig. 41A-C, Guiton *et al*, unpublished). Similar findings were obtained with the commensal *E. coli* strain MG1655 (data not shown). On the other hand, UPEC does not affect *E. faecalis* biofilm formation and virulence *in vivo* when introduced at a 1:1 ratio in implanted animals (Fig. 41D-E;

Guiton, P.S. *et al*, unpublished). Both bacterial species are recovered on the surface of implants and organs to the same extent as in single infections. Similar results were obtained in the kidneys (data not shown). Together, this indicates that the molecular dynamics of *in vitro* and *in vivo* biofilm formations in mixed communities are different. Identification of the *E. coli in vitro* inhibitory factor(s) may represent a target to prevent *in vivo* infections. Studies from the K2 pyelonephritis isolate CFT073 identified an inhibitory polysaccharide fragment[301]. However, UTI89 and MG1655 produce K1 and K12 capsules, respectively[302,303], suggesting the inhibitory factor identified in the present study is unique. It will be of interest to understand why UPEC does not inhibit enterococcal biofilms *in vivo*. Can it be that the anti-biofilm substance(s) is/are not produced in the urinary tract, produced and degraded, or present in too minute quantities *in vivo* to be effective against *E. faecalis* biofilms? Further research on this topic will contribute to a better understanding of the interactions between UPEC and *E. faecalis* and provide insight into potential novel anti-biofilm agents against enterococcal biofilms.

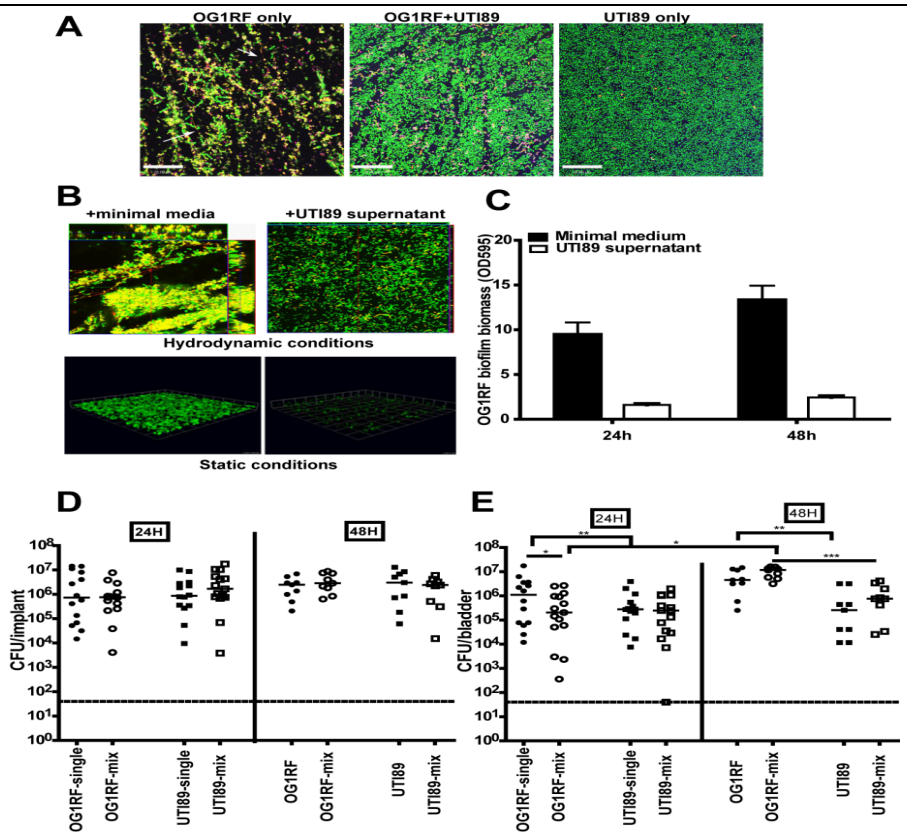

Figure 40: Differential interactions between *E. faecalis* and UPEC *in vitro* and *in vivo*.

Representative CLSM images of **(A)** single and mixed OG1RF and UTI89 dynamic biofilms in TSBG and **(B)** of OG1RF shaking and static biofilms grown in TSBG supplemented with minimal media (M63+niacin+glycerol) or filter-sterilized UTI89 supernatants from stationary cultures in minimal media grown for 48h on PVC coverslips at 37ºC. SYTO89 and PI were used to stain bacterial DNA in all experiments. In mixed biofilms, OG1RF was stained additionally with WGA (pink punctuates in middle panel in **A**). **(C)** Crystal violet based quantification of 24h OG1RF biofilms grown in TSBG supplemented with either minimal media or UTI89 supernatant. Error bars =SEM from two independent experiments. **(D-E)** Graphs represent bacterial titers in log scale from implants and bladders of animals inoculated for 24 and 48h with OG1RF (●), UTI89 (■), or a mixture of OG1RF (○) and UTI89 (□) at a 1:1 ratio. Horizontal dashed lines represent LOD for viable bacteria. The horizontal bars represent the median of each dataset; *p<0.05, **p<0.0005, ***p<0.0005, ns corresponds to p>0.05 by the Mann Whitney U test.

Implications for bladder cell biology and physiology:

The presence of a foreign body within the urinary tract alters the bladder environment and homeostasis, resulting in cellular proliferation, urothelial damage, increased exfoliation, and the induction of a massive inflammatory response. All these detrimental biological changes may lead to bladder carcinogenesis, as in the case of patients with spinal cord injuries who are at higher risk of developing bladder cancer following urinary catheterization[27,180,181]. Important questions regarding the rate of bladder exfoliation, urothelial cell division and differentiation, and the epithelial responses to urinary catheterization could possibly be addressed by comparing responses following bladder implantation in animals with different genetic backgrounds. Further research may be warranted to investigate the development of bladder tumors following urinary catheterization. Simply, to start, the effects of urinary catheterization could be assessed on the production and function of several known markers of bladder cancer, such as RhoGDI2[304] and Oct-3/4[305]. Additionally, further optimization of the murine model of CAUTI is required to ensure long term catheterization that may provide a novel tool for the study of urothelial tumorigenesis and metastasis in mice, similar to a previous model developed by Reis *et al*, but without involving the transurethral introduction of human carcinoma cells as in the orthotopic models of bladder cancers[306] or administration of chemicals such as organic arsenicals in the bladder[307,308]. Understanding the consequences of urinary catheterization on bladder homeostasis may provide insights into the damage and side effects resulting from this necessary medical process.

Clinical implications

The findings from this work question the very answer to "What is a UTI?" The current clinical definition refers to UTI as "the presence of bacteria within the urinary tract"[5] and even with the different classifications of UTIs, undoubtedly does not encompass all the variations and presentations the disease can take. CAUTIs are rarely symptomatic; yet, bacteriuria, which is almost inescapable following urinary catheterization[12], and pyuria. Pyuria is not a good correlate of CAUTIs[22]. The establishment of intracellular niches in the presence of urinary catheters is a major discovery in the pathophysiology of UPEC-mediated CAUTI (Chapter three). Thus, bacterial counts equal to or greater than 1000 CFU/ml and pyuria, which occurs regardless of microbial colonization, as demonstrated here, are not enough for defining appropriate treatment course for intracellular microbial populations. It is thus imperative to identify new diagnostic tools in order to have better preventative or therapeutic regimens for CAUTI patients. Work outlined herein establishes a new origin for catheter colonization, in addition to the intraluminal and extraluminal portals[178], implying that patients with history of UTIs may be at greater risk of CAUTI and rUTIs after catheter removal. The favored CAUTI treatment strategy, which is the removal of contaminated urinary catheter following bacteriuria, should be reconsidered in light of the findings presented here. Intracellular bacteria or pre-existing reservoirs from previous UTIs can resurface and cause recurrent infection even in the absence of catheters. It is thus imperative to re-examine the paradigms for diagnosing and treating CAUTIs.

In addition to leukocyte esterase and nitrite currently used as biomarkers of UTIs[309], it will be very advantageous to have additional host and bacterial biomarkers

that provide more insights into the type of infection and the stages of the disease. These biomarkers may include small molecules produced by multidrug resistant bacteria or the host immune cells during infections and biomaterials shed from biofilms during infections that can be detected in the urine. Bacterial DNA, polysaccharide fragments from the extracellular matrix of biofilms or particular proteins from the host secreted only in the presence of particular bacterial species may be among these biomarkers. Furthermore, this thesis shows that immunosuppression favors enterococcal infections, since dexamethasone treatment and neutrophil depletion enhance infections; corroborating the predominance of CAUTI among immunosuppressed and immunocompromised individuals[5]. This finding also implies that immunosuppression in a patient undergoing urinary catheterization should not be a favored approach in trying to reduce the inflammation and other side effects ensuing from catheterization or during the treatment regimens against CAUTIs.

Drug development

CAUTIs originate primarily from multidrug resistant bacteria. The identification of intracellular populations, as in the case of UPEC CAUTI coupled with the ever-increasing antibiotic resistance, especially within biofilms, underscores the need for better preventative and therapeutics approaches. As demonstrated in this dissertation, mannosides, which specifically target the FimH adhesin of UPEC, prevented the establishment of IBCs and, in combination with existing antibiotic-based therapy, prevented the establishment of implant and organ colonization in the murine model of CAUTIs. Thus, virulence-based approaches in addition to antimicrobial-coated catheters or bacterial interference approaches as previously proposed[293,310] can be novel avenues of

research for preventing and treating CAUTI. Surface proteins, such as LPxTG motif-containing cell wall proteins in Gram positive bacteria, are also attractive targets for virulence-based approaches as they are readily accessible, important for binding to abiotic surfaces as well as urothelial cells, and can be mediators of bacterial immune evasion.

Studies in this thesis also demonstrated that urinary catheterization induces urothelial damage and significant reduction in UPEC IBC formation. It is thus plausible that the use of abiotic irritants that induce urothelial exfoliation and/or release anti-biofilm agents such as mannosides in a controlled manner may be an effective treatment for women suffering from chronic and recurrent UTIs. It will be of interest to evaluate chemicals or devices that can enhance bladder exfoliation to remove intracellular reservoirs without favoring microbial colonization and causing further damage to the bladder. Such strategy may be helpful to reduce the rate of recurrent infections.

CONCLUDING REMARKS

CAUTIs are major nosocomial infections afflicting humans of both sexes and all ages and associated with important medical and societal burdens. The present dissertation provides a better understanding of these infections, particularly caused by UPEC and *E. faecalis*, using an optimized murine model of CAUTI. The study presented here describe important characteristics of CAUTIs, raise important questions regarding diagnostic approaches and propose novel preventative and therapeutic approaches against CAUTIs based on the identification and targeting of important biofilm determinants and virulence factors. The implications of the current report underscore the need for more research and

increased clinical attention to CAUTIs for a better management of these infections and ultimately the patients' welfare.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All strains used in this study and their characteristics are listed in Table 3.

For *E. faecalis:* Unless otherwise specified, all experiments were initiated from an overnight culture in brain heart infusion broth (BHI; Becton Dickinson, Franklin Lakes, NJ) of a single bacterial colony grown on BHI agar plate supplemented with the appropriate antibiotics. Liquid cultures were grown statically at 37°C for 18 hours.

For *E. coli*: bacteria from a single colony from Luria Bertoni (LB, Becton Dickinson) agar plate supplemented with the appropriate antibiotics were inoculated into LB broth and grown statically at 37°C for 18 hours or 2 x 24h.

Antibiotics used for bacterial selection: Rifampicin (Rif), Fusidic acid (Fus), Kanamycin (Kan), Chloroamphenicol (Cm), and Streptomycin (Str); from Sigma-Aldrich, Inc., St. Louis, MO.

Genetic manipulations and sequencing

Table 4 lists the plasmids used in this study.

For *E. faecalis*: In-frame deletion constructs were made in temperature sensitive plasmid pJRS233[311,312] for genes of interest based on the complete genome of *E. faecalis* V583[313]; all references to genomic loci below are based on this annotation (GenBank accession number AE016830). Chromosomal integrants were selected as previously described[314] with primers listed in Table 5. Complementation of indicated genes was done using a pABG5-derived pAL1 plasmid expressing the gene of interest under the *rofA* promoter[312,314,315].

For *E. coli*: Genetic mutations were generated as previously in described using λ Red Recombinase system[316,317].

DNA sequencing and sequence manipulations

Genes of interest were separately amplified in PCR reactions using OG1RF and OG1X colonies or purified genomic DNA as template. The primers used for amplification are listed in Table 7. Forward (F1 or 2) primers were used for amplification of the upstream region and Reverse (R1) primers were for amplification of the downstream region of the genes of interest annotated according to the genome sequence of *E. faecalis* V583[313]. PCR were performed as described in Chen *et al*[318]. Products were purified using the QIAquick PCR purification kit according to manufacturer's guidelines (Qiagen) and submitted for capillary sequencing with primers listed in table 7 using an ABI 3730*xl* instrument (Genewiz, Inc.). Base calling and assembly was done using Phred and Phrad[319,320]. Sequences were aligned using CLUSTAL W[321] with default parameters and trimmed to the start and stop codons of genes as annotated in the OG1RF and V583 genome sequences[168,313].

Inhibitors and chemicals

The drug treatments used in this study, their relevant modes of action, and references of dosage and effectiveness are described in Table 7. Vehicles were saline or DMSO as indicated.

Mouse strains

Six to seven week-old female wild-type C57BL/6Ncr mice purchased from the National Cancer Institute (NCI) were used in this study. Six to seven week old female C57BL/6J, interleukin 6 mutant B6.129S-IL6^{tm1kopf}/J (referred to in this study as IL6-/-), Toll Like

Receptor 2 mutant B6.129-*Tlr2*^{tm1Kir}/J (referred to in this study as TLR2-/-), *Rag* B6.129S7-*Rag1*^{tm1Mom}/J mice were obtained from Jackson Laboratories. *MyD88*, *Trif,* and *cKit*-mutant female mice from Jackson Laboratories were bred at Washington University in St Louis. Experiments were performed following one-week adaptation in the animal facility. All studies and procedures were approved by the Animal Studies Committee at Washington University School of Medicine.

Cultivation and quantification of biofilms

Bacterial preparation for biofilms: Stationary phase bacteria from overnight cultures were diluted to OD600 of 0.2 in fresh growth medium followed by an immediate 1:100 dilution (1-2x10^6 CFU/ml) in biofilm media: TSBG composed of tryptic soy broth (TSB; Becton Dickinson, Sparks, MD) supplemented with 0.25% of glucose (Sigma-Aldrich), LB, or human urine. Urine was collected from healthy volunteers as approved by the Institutional Review Board of Washington University in St. Louis. Pooled urine were spun at 10,000 x g for 15min, filtered through 33μM filters, and, if necessary, stored at 4ºC for no more than 3 days. Mixed biofilms were grown in TSBG with bacterial strains mixed at a 1:1 ratio prior to media inoculation. Supernatants from stationary phase UTI89 cultures grown in minimal medium (M63 salts with niacin and glycerol) or LB under static conditions at 37ºC were spun at maximum speed, filter-sterilized, and were added to TSBG at 1:4 ratio (20% supernatant to 80%TSBG) prior to OG1RF inoculation. Supernatants from stationary cultures of OG1RF in BHI and sterile minimal medium, LB, and BHI were supplemented to TSBG when indicated and used as controls.

Coverslip-based assays: Biofilms were grown as described by Gilmore *et al.*[322] and modified as follow: 5ml of diluted cultures were placed in each well of sterile 6-well

tissue-culture flat bottom plates (Techno Plastic Products, St Louis, MO) containing a UV-sterilized 22x22mm plastic cover slip (Fisher scientific, Pittsburgh, PA). Plates were incubated at 37°C statically or under dynamic conditions (100RPM) on an orbit shaker (model 3520; Lab-Line Instruments, Inc., Melrose Park, IL) for the duration of the experiments. At 24hr intervals, media were removed by aspiration and the cover slips were washed twice with 5ml of autoclaved water for 3 minutes at 100RPM on orbit shaker at room temperature (22-25°C) and fresh media added. At selected time points, biofilms were washed and air-dried. Biofilms were then stained with 0.5% crystal violet (Sigma-Aldrich, Inc., St Louis, MO) for 10 minutes at room temperature. Excess dye was removed with autoclaved water and air-dried at room temperature. Biofilms were then dissolved with 500µl 33% acetic acid (Fisher Scientific, Fair Lawn, NJ). Biomass was quantified colorimetrically by measuring the absorbance at OD595 with a microplate reader (Molecular Devices, Sunnyvale, CA). Experiments were performed independently at least three times with three coverslips/condition/experiment.

Fluid flow-based assay: Biofilms were grown as described by Ferrieres *et al*.[323] on All Silicone Foley catheters (Bard Medical, GA) or silicone tubing (Thermo Fisher Scientific Inc.) and modified as follows. All tubing and connectors in the system were autoclaved and ethanol sterilized prior to use. The system was assembled similar to the previously described flow-chamber system. Priming of the catheter or the silicone tubing occurred at 37ºC for 20min by flowing pre-warmed pooled human urine. Pooled samples were spun at 10000xg for 15min, filtered through 33µM filters, and, if necessary, stored at 4ºC for no more than 3 days. Three milliliters of stationary-phase *E. coli* from overnight cultures were diluted to 1-2x10^6 CFU/ml in human urine and injected into the

catheter or silicone tubing using a 30 cc gauge needle. The bacteria were allowed to attach to the substratum for 1 h before urine flow via Watson-Marlow peristaltic pump 205S was resumed at 0.5 ml min^{-1}. When indicated, urine was supplemented with 1% methyl-α-D-mannopyranoside (Sigma-Aldrich) prior to the experiment. After 24 hours, the remaining medium was exchanged for sterile ddH2O that was allowed to flow at 0.5 ml $min^{-1}$to remove residual urine and non-adherent bacteria in the system. The liquid from catheter or silicone tubing was then removed by capillary action onto absorbent paper. The tubing was cut into pieces for CFU enumeration or crystal violet staining, respectively. For CFU enumeration, at least three pieces (1cm in length) of incubated tubing were separately further cut into smaller pieces and placed into 1ml PBS. Adherent cells were detached by sonication (10min) and vigorous vortexing (3min). Viable bacterial counts were assessed by serial dilution on BHI or LB agar plate with appropriate antibiotics. Crystal violet staining was used to determine biofilm biomass. At least 3 pieces of incubated tubing (3cm in length) were filled with 0.5% crystal violet at room temperature for 10 min. Excess dye was removed by washing three times with ddH2O and dried by capillary action on absorbent paper. The bound crystal violet was then dissolved in 200 µl of 33% acetic acid and absorbance measured at 595nm. The amount of biofilm was expressed as CFU/ml per cm^2 and A595/cm^2. The experiment was repeated at least twice with different urine samples.

Primary adherence assay

Biofilms were set up according to the coverslip-based assay described above. Cells were allowed to attach for 2h, 4h, and 6h at 37°C under static or shaking conditions. After

desired incubation times, cover slips were washed once with sterile water and processed as described above. Adherent cells were quantified by OD595 measurements.

DNase I assays

DNase I (Sigma Aldrich) or heat inactivated DNase I (30 minutes at 100°C) was added at a final concentration of 5µg/ml, unless otherwise stated in coverslip-based biofilm assays. Heat-inactivation of DNase I was verified by its inability to digest 100µg/ml of salmon sperm DNA or purified enterococcal genomic DNA in TSBG for 1h or 24h at 37°C. DNase I was either supplemented continuously every 24hrs from the beginning of the experiment or added to preformed biofilms for the time indicated. Experiments were performed independently in triplicate with three cover slips/condition/experiment.

Bacteria and biofilm visualization

By Confocal Laser Scanning Microscopy: All confocal microscopy was performed on an LSM 510 Meta Laser Scanning Confocal microscope (Carl Zeiss, Thornwood, NY), using a 63X oil immersion objective. Images were acquired using the LSM Image Examiner software (Carl Zeiss, Thornwood, NY). 3D reconstruction of biofilm images were generated using Volocity software (Improvision, Inc., Waltham, MA). Washed plastic cover slips were stained for 15 minutes in the dark at room temperature with 1ml of 3µM of SYTO9 mixed with 3µM of propidium iodide (PI) from Molecular Probes, Eugene, OR. When indicated, 1µg/ml of Alexa Fluor 633-conjuaged Wheat Germ Agglutinin (WGA) was added for carbohydrate staining[324]. Excess dye was removed by aspiration and biofilms were washed twice with 1X phosphate buffer saline (PBS) solution or water. Stained cover slips were mounted on glass slides using Prolong® Gold

Antifade (Molecular Probes) followed by image acquisition. Microscopy was performed on two coverslips/condition/experiment with at least two independent experiments.

***By Freeze Dry Electron Microscopy*:** Biofilms grown on glass cover slips in TSBG under hydrodynamic conditions for 48h on were fixed with 2% glutaraldehyde in 100mM NaCl, 30mM Hepes, 2mM $CaCl_2$ and 75mM Sucrose, pH 7.2 for 1 hour. Samples were rinsed in deionized water to remove aldehyde and buffer, and quick-frozen by forceful impact against a copper block cooled to 4°K with liquid helium. Frozen cultures were stored in liquid nitrogen until mounting in a Balzers 301 vacuum evaporator, wherein they were freeze-dried for 20 minutes at –80°C and then rotary-replicated with 2 nm of platinum deposited from an electron-beam gun mounted at 15 degrees above the horizontal. The Pt-replica was then stabilized with a carbon film deposited from a 70° angle. Replicas were floated off the glass onto concentrated hydrofluoric acid, then transferred through several rinses of deionized water and picked up on formvar-coated grids. Replicas were examined in a JEOL 100CX microscope and photographed with an AMT digital camera.

By Transmission Electron Microscopy: For immunolocalization of DNA at the ultrastructural level, 48h dynamic biofilms were fixed in 4% paraformaldehyde/0.05% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100mM PIPES/0.5mM $MgCl_2$, pH 7.2 for 1 hr at 4°C. Samples were then embedded in 10% gelatin and infiltrated overnight with 2.3M sucrose/20% polyvinyl pyrrolidone in PIPES/$MgCl_2$ at 4°C. Samples were trimmed, frozen in liquid nitrogen, and sectioned with a Leica Ultracut UCT cryo-ultramicrotome (Leica Microsystems Inc., Bannockburn, IL). 50 nm sections were blocked with 5% Fetal Bovine Serum (FBS)/5% Normal Goat Serum (NGS) for 30

min and subsequently incubated with mouse anti-double-stranded DNA (anti-dsDNA) monoclonal antibody (Abcam, Cambridge, MA) for 1 hr at room temperature. Sections were then washed in block buffer and probed with 18nm colloidal gold-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove PA) for 1 hr at room temperature. Sections were washed in PIPES buffer followed by a water rinse, and stained with 0.3% uranyl acetate/2% polyvinyl alcohol. Samples were viewed with a JEOL 1200EX transmission electron microscope (JEOL USA Inc., Peabody, MA). All labeling experiments were conducted in parallel with controls omitting the primary antibody. These controls were consistently negative at the concentration of colloidal gold conjugated secondary antibodies used in these studies. Quantification was based on gold particles labeling from 13 independent fields at 30000X magnification.

By Negative staining and analysis by electron microscopy: Bacteria were grown overnight in BHI and diluted as described above in TSBG. They were allowed to grow statically for 6h at 37°C. Cells were pelleted and resuspended in 1X phosphate buffered saline (PBS). Bacteria were allowed to absorb onto formvar/carbon-coated copper grids for 1 min. Grids were washed in dH_2O and stained with 1% aqueous uranyl acetate (Ted Pella Inc., Redding CA) for 1 min. Excess liquid was gently wicked off and grids were allowed to air dry. Samples were viewed on a JEOL 1200EX transmission electron microscope (JEOL USA, Peabody, MA) at an accelerating voltage of 80kV.

By Scanning Electron Microscopy: Biofilm grown on silicone tubing were fixed in 4% paraformaldehyde, 2.5% glutaraldehyde, and 0.1M cacodylate, and prepared for scanning electron microscopy (Hitachi Ltd, Tokyo).

Cellular autolysis assay

The autolysis assay was performed as previously described in sodium phosphate buffer [139]. Briefly, overnight cultures were inoculated in fresh TSBG at a final O.D. of 0.002 at 600nm and grown at 37°C on an orbit shaker at 100RPM to an O.D. of 0.6 at 600nm or after 24h. Cultures were then placed on ice for 10 min and 1.5ml were removed and centrifuged at 14,000 x g for 5min at 4°C. The supernatants were discarded and the pellets were resuspended in 1.5ml of ice cold water and washed thrice at 14,000 x g for 5min at 4°C. Subsequent to the third wash, the pellets were resuspended in 10mM sodium phosphate buffer at pH 6.8 supplemented with 0.5µg/ml of trypsin (Sigma-Aldrich). 200µl of the suspensions were dispensed in a 96-well flat bottom microtiter plates in quintuplet and the remaining wells were filled with ddH_2O. Samples were incubated at 37°C and O.D. readings were taken every 30 minutes for up to 9 hours at 600nm in a spectrophotometer. Autolysis was expressed as percent of initial turbidity at 600nm.

Murine implantation procedure

The experimental model for foreign-body associated UTI [29,194] was adapted for the study of *E. faecalis* and *E. coli* with the following modifications. Polyethylene tubing (PE10 outside diameter, 0.6mm, inner diameter, 0.28mm; Becton Dickinson, MD) and platinum-cured silicone tubing (SIL025: outside diameter, 0.64mm; inside diameter, 0.30mm; Braintree Scientific, Inc, MA) were sterilized with 70% ethanol and air-dried. A seven-millimeter-long segment of PE10 was fitted onto a 30.5-gauge sterile needle (Becton Dickinson). A four to five millimeter-long straight segment of the SIL025 was then placed on top of the PE10 segment. The assembly was then UV-sterilized for 24h.

Seven to eight week-old female wild-type C57BL/6Ncr mice were anesthetized with inhaled isoflurane and placed on their backs. The periurethral area was sterilized with 100% ethanol and 10% povidine-iodine solution. The needle with both PE10 and SIL025 was gently inserted in the urethral opening. The long segment (PE10) was delicately advanced with tweezers until the shorter segment (SIL025) was released into the bladder. Subsequently, the needle with the PE10 was removed leaving the silicone implant in the murine bladder. The recovery of straight silicone implants was greater than 95% at 24 hours and approximately 75% seven days following implantation.

Bacterial infection and colony forming unit (CFU) determination

Bacterial cells were collected from overnight cultures by centrifugation at 8,000 X g for 5 min and resuspended in 1x phosphate-buffered saline (PBS) to an approximate optical density at 600nm of 1 corresponding to approximately $7x10^8$ CFU/ml. When indicated, bacteria were further diluted to OD600 of 0.5. Seven- to eight- week-old female C57BL/6Ncr mice with or without silicone implant were anesthetized by inhalation of isoflurane and infected by transurethral catheterization as previously described[62,325] with 50µl inocula of PBS or bacterial suspensions (~1-3.5x10^7 CFU) immediately following implantation. For polymicrobial infections, bacterial species were mixed at a 1:1 ratio to reach (~1-2x10^7 CFU/strain). To quantify bacteria present on implants and in mouse organs, mice were sacrificed at desired time points by cervical dislocation after anesthesia inhalation, and the bladders and kidneys were aseptically harvested. Subsequently, the silicone implant was retrieved from the bladder when present, placed in PBS, sonicated for 10 min and then vortexed at maximum speed for 3 min. Bladder and pairs of kidneys from each mouse were homogenized in PBS. For bacterial enumeration

on implants and tissues, samples were serially diluted and plated on BHI supplemented with the appropriate antibiotics or LB plates. Colony forming units (CFU) were enumerated after 24h incubation at 37˚C. Experiments were performed at least twice with n=5 mice/strain/condition. Biofilms on retrieved implants were visualized by scanning electron microscopy at indicated time points.

Bladder weight determination and plasma protein extravasation

When indicated, bladders were aseptically removed, bisected, and blotted dry. Bladders were placed in pre-weighed eppendorf tubes and weighed. Bladder weight (in grams) was determined as the difference between bladder and eppendorf tube weight and that of the empty eppendorf tube.

Plasma protein extravasation (PPE) in the bladder was determined using the Evans blue technique[326] in non-implanted and implanted animals at 6h post treatments with indicated chemicals listed in Table 6. Mice were anesthetized by inhalation of isoflurane and 30mg/kg Evans Blue (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was administered intravenously (i.v.). Animals were sacrificed 15 min later by cervical dislocation. The bladder was then excised, bisected, and blotted dry before weighing. Bladders were then placed in 1ml of 100% formamide (Sigma-Aldrich) and incubated at 60ºC for 24 h for Evans blue extraction. The extracted Evans blue from each bladder was quantified by colorimetric measurement at 620 nm with an automated microplate reader. The dye content extravasated in each bladder was determined from an Evans blue standard curve and expressed as μg/g of tissue.

Mass spectrometry analysis

To assess the presence of substance P following urinary catheterization, non-implanted and implanted animals with and without bacterial infections (one/experimental group) were sacrificed 3hpi. Bladders were retrieved, homogenized, spun at maximum speed to pellet cellular debris, and bladder homogenates were frozen at -80ºC until further use.

Substance P is an undecapeptide with a molecular weight of 1347.6 and synthetic Substance P was purchased from Santa Cruz Biotechnology and used as positive control. Under routine electrospray conditions described below the 2+ and 1+ ions at 674m/z and 1347m/z respectively dominate the spectrum. The peptide does not fragment to yield a nice sequence of ions but rather only seems to lose H_2O and the C-terminal methionine. The ms2 spectrum has two major ions m/z 600, which is the b_{10}^{2+} and m/z 666 the b_{11}^{2+}. The y_9 (m/z 1095) and b_2 (m/z 254) are also present to a lesser extent. This is in agreement with the literature[327].

C_{18} SPE columns (Waters, Milford, MA) were used to cleanup bladder homogenates and synthesized Substance P control. Substance P elutes in a 50% MeOH fraction. Samples were then applied to the SPE columns and the columns were washed (W) and then eluted (E). SRM scans were run on all W and E fractions but Substance P was not detected.

The mass spectrometer used for the study was a Thermo-Finnigan LCQ Deca coupled to a Waters capLC. Samples were injected onto a Vydac C18 MS column (150mm 0.3mm X 5u) with a flow rate of 6ul/min. At time 0 Solvent A (0.1% formic acid) was held at 92% and solvent B (80% acetonitrile in 0.1% formic acid) at 8%. Initial conditions were held constant for 5 minutes, before beginning a linear gradient which increased to 85% B in the next 45 minutes. The column was allowed to equilibrate for 15 minutes before

starting the next run. The spray voltage on the spectrometer was 4.5K and the capillary temperature was 200 ºC. For CID experiments helium was used as the collision gas with the collision energy set to 30% of the maximum (~5 eV). Spectra were recorded from m/z 216-2000 in the positive centroid mode. Ions were monitored with a window of +/- 0.5amu

Cytokine profiling

Bladder homogenates from non-implanted and implanted animals with or without bacterial infections were microcentrifuged at 14,000 r.p.m. for 5 min and supernatants frozen at −80°C until the time of the assay. Assays were carried out according to manufacturers' protocols using the Bio-Plex Pro Mouse Cytokine 23-plex Assay kit from Bio-Rad Laboratories (Hercules, CA).

Histopathology and immunohistochemistry

For histological analyses, bladders were fixed in methacarn (60% methanol, 30% chloroform, and 10% glacial acetic acid) or formalin for 1-2 h at room temperature and dehydrated in 70% ethanol overnight at 4ºC. They were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H/E) for light microscopy. For immunofluorescence examination, unstained bladder sections were deparrafinized with xylene (twice for 10 min), rinsed in 100% isopropanol (three times for 5 min), and washed with running de-ionized water for 5 min. When indicated, antigen retrieval was performed in Citrate Buffer (10mM NaCitrate pH 6.0) as previously described[328,329]. Slides were washed in PBS for 5 min and in blocking buffer (5% Fetal Bovine Serum or PBS with 1%BSA and 0.3% TritonX-100) for 1h at room temperature. This step was followed by a PBS wash (three times for 5 min). The tissue sections were then blocked

statically in blocking buffer for 30 min at room temperature. Primary antibodies, raised in rabbit against *Streptococcus* Group D antigen (Lee Laboratories), against human nitric oxide synthase 2 (NOS2 or iNOS; Santa Cruz Biotechnology), against rat NK-1 receptor (Pierce Biotechnology, Rockford, IL), raised in goat against mouse uroplakin III (Santa Cruz Biotechnology, Inc., CA) and against human substance P (Santa Cruz Biotechnology), were added in blocking buffer at 1:500 dilution when indicated and incubated at room temperature for 1 hour. After a PBS wash (three times for 5 min), tissue sections were incubated with secondary antibody, Alexa Fluor 555-labeled donkey anti-rabbit antibody and Alexa Fluor 488-labeled donkey anti-goat antibody, each diluted 1:1000 in blocking buffer for 30 min at room temperature. After PBS wash (once for 5 min), tissues were counterstained with TOPRO-3 (Molecular Probes) or Alexa Fluor 633-conjugated WGA at 1:1000 dilution in PBS (second wash for 5 min) to reveal nuclear morphology, when indicated. After a third PBS wash for 5 min, immunostained tissues were mounted using Prolong Gold Antifade (Molecular Probes) and visualized by CLSM.

Flow cytometry

Single cell bladder suspensions were made from minced bladder tissues subjected to collagenase/DNAse I digestion. Staining of surface markers was performed using FcR block and fluorochrome-conjugated monoclonal antibodies (mAbs).

To immunophenotype the immune infiltrates, specific combinations of mAbs were chosen which distinguish granulocytes ($CD11b^{+}Gr1^{+}Ly6G^{+}$), monocytes ($Ly6G^{-}Ly6C^{+}$), macrophages ($CD11b^{+}F4/80^{+}$), dendritic cells ($CD11c^{+}F4/80^{-}$), basophils ($FccR1^{+}$), eosinophils ($SiglecF^{+}$), mast cells ($cKit^{+}$), NK cells ($NK1.1^{+}$), T cells ($CD3^{+}CD4^{+/-}CD8^{+/-}$), and B cells ($CD19^{+}$). All antibodies were from BD Pharmagen,

Ebioscience or Southern Biotech. Cells were counterstained with propidium iodide. Activation status was determined using specific mAbs for MHC-II. Samples were acquired on a FACScalibur (BD Biosciences) and data were analyzed using FlowJo software (version 7.6.4). The absolute number of cellular infiltrates per bladder was calculated by multiplying the percentage of each population from two parameter flow cytometry data by the total number of isolated live cells, as determined by hemocytometer count following Trypan blue staining.

Neutrophil depletion

Mice were rendered neutropenic as previously described [219]. Briefly, an anti-Ly6G mAb (1A8) from (Bio X Cell, West Lebanon, NH) was administered intraperitoneally on days 3 and 1 prior to implantation and bacterial challenge. Control mice received IgG isotype control 2A3 (Bio X Cell) in the similar manner.

Gentamicin protection assay

To quantify intracellular and extracellular bacteria, bladders were aseptically harvested at indicated time points. Bladders were cut in 4 parts and washed three times in 500 μl PBS. The wash fractions were pooled, centrifuged at 500 rpm for 5 min to pellet exfoliated bladder cells. The supernatants were then serially diluted and plated on LB agar supplemented with appropriate antibiotics to obtain the extracellular bacterial contents. The dissected bladders were treated with 100μg/ml gentamicin (Sigma-Aldrich) for 90 min at 37^{o}C. Following gentamicin treatment, the bladder tissue was washed twice with PBS to eliminate residual antibiotics, homogenized in 1 ml PBS, serially diluted and plated on LB agar with appropriate selection antibiotics for quantification of the intracellular bacteria. CFU was enumerated after 24 h of growth at 37^{o}C.

UPEC reservoir reactivation

Non-implanted animals were infected with UTI89HK::GFP as described above. At fourteen days post infection, urine was collected, serially diluted and plated for CFU and a subset of animals implanted as described above. Animals determined to be bacteriuric (bacterial loads greater than or equal to 10^4 CFU/ml in urine) as counted on titer plates the next day were eliminated from further study. UPEC reservoir reactivation post-implantation was assessed by CFU enumeration of bacteria on implants and in the organs 3 or 5 days post-implantation (17 or 19 dpi). UTI89HK::GFP titers greater than 10^4 CFU/ml on implants or bladders were considered reactivation events. Measures of reactivation events of animals, which were non-bacteriuric at 14 dpi but non-implanted served as controls.

Mannoside and antibiotic treatment

For pretreatment experiments, 50µl mannoside (ZH56; 5 mg/kg mouse body weight) or PBS was administered intraperitoneally 30min prior to implantation as previously described[87]. As indicated for pre-infection treatment, trimethoprim/sulfamethoxazole (TMP-SMZ) was added at 54 and 270 µg/ml, respectively, to the drinking water for three days prior to bacterial inoculation. The drinking water was changed every 24h.

To assess the effects of mannoside and/or TMP-SMZ on established infections, animals were implanted and infected for 24h. At 24hpi, TMP-SMZ was added to the drinking at the concentrations indicated above and ZH56 or PBS was administered i.p. 6h prior to sacrifice. Animals were sacrificed 48hpi.

IBC enumeration and visualization

Implanted and non-implanted animals were infected with UTI89 for 6h. When indicated, ZH56 (5mg/kg) or PBS was administered i.p. at 30 min prior to implantation. At 6hpi, bladders were harvested, bisected, splayed on silicone plates and fixed in 2% paraformaldehyde. LacZ staining of whole bladders was performed as previously described[330]. Punctate violet spots characteristic of IBCs were enumerated by light microscopy.

For IBC visualization, animals were infected with UTI89 constitutively expressing GFP (UTI89pCom-GFP). At the indicated time point, bladders were removed, bisected, splayed, and fixed as described above. The splayed bladders were then incubated for 20 min at room temperature with Alexa Fluor 633-conjugated wheat germ agglutinin (WGA; 1:1000 in PBS; Molecular Probes) for staining of the bladder surface and, when indicated, SYTO83 (1:1000 in PBS; Molecular Probes) to stain bacteria. Bladders were rinsed with PBS, mounted using Prolong Gold antifade reagent (Invitrogen) and examined with a Zeiss LSM510 confocal laser-scanning microscope under a 63X objective. SYTO83 and WGA were excited at 543 and 633 nm, respectively.

Statistical methods

Comparisons between groups were conducted by nonparametric two tailed, Mann-Whitney U test using GraphPad Prism (GraphPad software). A *p*-value less than 0.05 was considered significant. For *in vivo* experiments, values below the limit of detection (LOD) were assigned the appropriate LOD value for statistical analyses. Colonization and infection was defined as organs/implants with bacterial titers above the limit of detection at 24 hpi.

Table 3: List of strains used in this study

Species and Strains	Relevant Antibiotic resistance	Characteristics	References
E. faecalis OG1RF WT	Rif^R, Fus^R	Parental enterococcal strain, derived from the oral isolate from OG1	209
OG1RFΔ*atn*	Rif^R, Fus^R	OG1RF with an in-frame deletion of *atn*, autolysin deficient	This study [210]
OG1RFΔ*srtA*	Rif^R, Fus^R	OG1RF with an in-frame deletion of *srtA*, SrtA deficient	This study [210]
OG1RFΔ*srtA/srtA*	Rif^R, Fus^R, Kan^R	OG1RFΔ*srtA* ectopically expressing a wild type copy of *srtA*, SrtA positive	This study [210]
OG1RF Δ*gelE*	Rif^R, Fus^R	OG1RF with an in-frame deletion of *gelE*, gelatinase deficient	103
OG1RF Δ*eep*	Rif^R, Fus^R	OG1RF with an in-frame deletion of *eep*, Eep defective	This study, Frank *et al*, in preparation
OG1RF Ω*ef0983*	Rif^R, Fus^R, Cm^R	OG1RF with a transposon insertion in *ef0983*, AhrC defective	This study [114]
E. faecalis OG1X	Str^R	*gelE* negative from nitrosoguanidine mutagenesis	209, 331
UTI89		Parental UPEC UTI89 strain, cystitis isolate	282
UTI89Δ*fimH*		UTI89 with an in-frame deletion of *fimH*, type 1 pili defective	75
UTI89Δ*sfaA-H*		UTI89 with an in-frame deletion of *sfa* operon, S pili defective	This study, Wright *et al*, unpublished
UTI89Δ*sfaA-H*Δ*fimB-H*	Kan^R Cm^R	UTI89 with in-frame deletion of the *sfa* operon and the fim operon from *fimB* to *fimH*, S and type1 pili defective	This study Wright *et al*, unpublished
UTI89Δ*csgA*		UTI89 with an in-frame deletion of *csgA,* curli deficient	80
UTI89Δ*csgB*Δ*csgG*		UTI89 with in-frame deletions of *csgB* and *csgG,* curli deficient	80
UTI89HK::GFP	Kan^R	UTI89 with an insertion of kanamycin cassette and green fluorescent protein (GFP) at the HK site	292
UTI89pCOMGFP	Kan^R	UTI89 ectopically expressing GFP	292

Table 4: List of plasmids used in this study

Name	Relevant Antibiotic resistance	Characteristics	References
pABG5	Kan^R, Cm^R (*E. coli*) Kan^{500}, Cm^{20} (*E. faecalis*)	Shuttle vector, containing *rofA* promoter for expression in Gram Positive bacteria	[332]
pJRS233	Erm^{750} (*E. coli*) Erm^{25} (*E. faecalis*)	Temperature sensitive plasmid for generation of in-frame deletions	[311, 312]
pAL1	Kan^{50} (*E. coli*) Kan^{500} (*E. faecalis*)	Derivative of pABG5, Cmr cassette, contains *rofA* promoter	This study [210]
pAL1::SrtA	Kan^{50} (*E. coli*) Kan^{500} (*E. faecalis*)	For complementation of *srtA* deletion	This study [210]
pCOM-GFP	Kan^{50} (*E. coli*)	For expression of GFP	[292]

Table 5: List of primers used in this study

Primer	Sequence (5′-3′)	Use	Reference
EF0799f1	GCTCTAGAAAGTTGCAGCTCCGGCAATGCCA CAACCGACG	Amplification of upstream region of *atn*	This study [210]
EF0799sewr	GTTACCAAAGATAGCTATTTTTTTCAATCTTA AATTAACCAACTTTCGTCCCCAACTTTCCTTT TTTAACTTATATGT	Amplification of upstream region of *atn*	This study [210]
EF0799sewf	GTTACCAAAGATAGCTATTTTTTTCAATCTTA AATTAACCAACTTTCGTCCCCAACTTTCCTTT TTTAACTTATATGT	Amplification of downstream region of *atn*	This study [210]
EF0799r1	GCTCTAGAACCCAGTTAACCGCAACTTCTGCT TCATAAGC	Amplification of downstream region of *atn*	This study [210]
EF0799f2	ATTTGCCACCTGCAGCCATT	Screening of *atn* deletion	This study [210]
EF0799r2	CCGCTCCCTCTTTCTACACG	Screening of *atn* deletion	This study [210]
EF3056e-f1	GGAATTCCCCCTCTACTAGCCTCCTTACCATT TTAC	Screening of *srtA* deletion	[314]
EF3056e-r1	GGAATTCCGTTGATAATGAGTCTGCCGCTAG TGTATG	Screening of *srtA* deletion	[314]

Table 6: Primers for confirmation of SNPs between OG1RF and OG1X

Primer	Sequence (5′-3′)
EF0243F1	GGT TGC TCT CTG TTT CTT CTT CTG
EF0243R1	CGA CAC TAG AAA AGA TGC GCT TCC
EF1218F1	GTG TTG AAA TAT ACG TTT TCC TTG CG
EF1218R1	AAG TCA CCA CAA TCA AAC ATA ATG G
EF1349F1	CG ACA GAT GTC ACT GCT TTA GC
EF1349R1	GTC TCT CGG TTG TTG AAG TTC C
EF1778F1	CAG AGA AAA TTC ATG CTC TTG AGC
EF1778R1	TCG CTG TAA TTT TTT GGC GAT TGC
EF2575F1	CAT GTG CGT TGA CTT CAT GCA ACG
EF2575R1	GTG AAT AAG GTC CTA CAG CAG C
EF2587F1	CTC AAT ACT AAC TCA AGT CAT AGC
EF2587R1	GGT ACT GCC AAG GTA ATT TAA AGC
EF2983F1	CAG GAT GAC CTC TAA AAA CCA TGC
EF2983R1	CAC TAC GTA CAG CAT TTT TCC ACC
EF3157F1	GCA AGC GGT GAT TCA AGC ATT CG
EF3157F2	CTG ACA CAG CGA AAC AAT TGT TGC
EF3157R1	CCA AGC TTG ATA ATG AAA TTT GGC
EF3285F1	GTC GTA TTT ACA TCA GAA AGC G
EF3285R1	ACC AAC TCG GGC ACC ATC G
EF3290F1	AGA TAA GAT TGA AGA AGC AAC TGG
EF3290R1	CAA ACT CTC TGG AAT TCA GTG C

Table 7: List of inhibitors and antibiotics used in this study

Name	Dosage/route	Mode of action	Company/References
Dexamethasone Sodium Phosphate	10mg/kg, i.p.30min prior to implantation	Glucocorticoid, anti-inflammatory and immunosuppressant	American Regent, Inc., Shirley, NY [245]
CP-99,994 dihydrochloride	5-10mg/kg, i.p., i.v., s.c.30 min and 3h post implantation (when indicated)	High affinity neurokinin 1 receptor (NK1R) antagonist	Tocris Bioscience, Ellisville, MO [201,202]
CP-96,345	5-10mg/kg, i.p., i.v., s.c, 30 min and 3h post implantation (when indicated)	High affinity NK1R antagonist	Tocris Bioscience [199]
Aminoguanidine hydrochloride	200mg/kg 1h and 3h post implantation (when indicated)	Irreversible inducible nitric oxide synthase (iNOS) inhibitor	Tocris Bioscience [205]
Cyclophosphamide	150mg/kg at time of infection	Chemotherapeutic agent/prodrug	Tocris Bioscience [333,216]
Alfuzosin hydrochloride	60µg/kg, 15min prior to implantation	α_1 adrenoreceptor antagonist	Tocris Bioscience [206,334]

REFERENCES

1 Griebling, T. L. Urologic diseases in America project: trends in resource use for urinary tract infections in women. *The Journal of urology* **173**, 1281-1287, doi:10.1097/01.ju.0000155596.98780.82 (2005).

2 Foxman, B. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. *Disease-a-month : DM* **49**, 53-70, doi:10.1067/mda.2003.7 (2003).

3 DeFrances, C. J., Lucas, C. A., Buie, V. C. & Golosinskiy, A. 2006 National Hospital Discharge Survey. *National health statistics reports*, 1-20 (2008).

4 Griebling, T. L. Urologic diseases in america project: trends in resource use for urinary tract infections in men. *The Journal of urology* **173**, 1288-1294, doi:10.1097/01.ju.0000155595.98120.8e (2005).

5 Foxman, B. The epidemiology of urinary tract infection. *Nature reviews. Urology* **7**, 653-660, doi:10.1038/nrurol.2010.190 (2010).

6 Foxman, B., Barlow, R., D'Arcy, H., Gillespie, B. & Sobel, J. D. Urinary tract infection: self-reported incidence and associated costs. *Annals of epidemiology* **10**, 509-515 (2000).

7 Foxman, B. *et al.* Risk factors for second urinary tract infection among college women. *American journal of epidemiology* **151**, 1194-1205 (2000).

8 Hooton, T. M. *et al.* A prospective study of risk factors for symptomatic urinary tract infection in young women. *The New England journal of medicine* **335**, 468-474, doi:10.1056/NEJM199608153350703 (1996).

9 Ikaheimo, R. *et al.* Recurrence of urinary tract infection in a primary care setting: analysis of a 1-year follow-up of 179 women. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **22**, 91-99 (1996).

10 Scholes, D. *et al.* Risk factors for recurrent urinary tract infection in young women. *The Journal of infectious diseases* **182**, 1177-1182, doi:10.1086/315827 (2000).

11 Huang, H. P., Lai, Y. C., Tsai, I. J., Chen, S. Y. & Tsau, Y. K. Renal ultrasonography should be done routinely in children with first urinary tract infections. *Urology* **71**, 439-443, doi:10.1016/j.urology.2007.10.049 (2008).

12 Tambyah, P. A. & Maki, D. G. Catheter-associated urinary tract infection is rarely symptomatic: a prospective study of 1,497 catheterized patients. *Archives of internal medicine* **160**, 678-682 (2000).

13 Jain, P., Parada, J. P., David, A. & Smith, L. G. Overuse of the indwelling urinary tract catheter in hospitalized medical patients. *Archives of internal medicine* **155**, 1425-1429 (1995).

14 Parker, D. *et al.* Catheter-associated urinary tract infections: fact sheet. *Journal of wound, ostomy, and continence nursing : official publication of The Wound, Ostomy and Continence Nurses Society / WOCN* **36**, 156-159, doi:10.1097/01.WON.0000347656.94969.99 (2009).

15 Jarvis, W. R. Selected aspects of the socioeconomic impact of nosocomial infections: morbidity, mortality, cost, and prevention. *Infection control and*

hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America **17**, 552-557 (1996).

16 Tambyah, P. A., Knasinski, V. & Maki, D. G. The direct costs of nosocomial catheter-associated urinary tract infection in the era of managed care. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America* **23**, 27-31, doi:10.1086/501964 (2002).

17 Hooton, T. M. *et al.* Diagnosis, prevention, and treatment of catheter-associated urinary tract infection in adults: 2009 International Clinical Practice Guidelines from the Infectious Diseases Society of America. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **50**, 625-663 (2010).

18 Maki, D. G. & Tambyah, P. A. Engineering out the risk for infection with urinary catheters. *Emerging infectious diseases* **7**, 342-347 (2001).

19 Stensballe, J. *et al.* Infection risk with nitrofurazone-impregnated urinary catheters in trauma patients: a randomized trial. *Annals of internal medicine* **147**, 285-293 (2007).

20 Lo, E. *et al.* Strategies to prevent catheter-associated urinary tract infections in acute care hospitals. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America* **29 Suppl 1**, S41-50, doi:10.1086/591066 (2008).

21 Trautner, B. W. Management of catheter-associated urinary tract infection. *Current opinion in infectious diseases* **23**, 76-82, doi:10.1097/QCO.0b013e328334dda8 (2010).

22 Tambyah, P. A. & Maki, D. G. The relationship between pyuria and infection in patients with indwelling urinary catheters: a prospective study of 761 patients. *Archives of internal medicine* **160**, 673-677 (2000).

23 Colgan, R. & Williams, M. Diagnosis and treatment of acute uncomplicated cystitis. *American family physician* **84**, 771-776 (2011).

24 Warren, J. W. Catheter-associated urinary tract infections. *Infectious disease clinics of North America* **11**, 609-622 (1997).

25 Goble, N. M., Clarke, T. & Hammonds, J. C. Histological changes in the urinary bladder secondary to urethral catheterisation. *British journal of urology* **63**, 354-357 (1989).

26 Delnay, K. M., Stonehill, W. H., Goldman, H., Jukkola, A. F. & Dmochowski, R. R. Bladder histological changes associated with chronic indwelling urinary catheter. *The Journal of urology* **161**, 1106-1108; discussion 1108-1109 (1999).

27 Vaidyanathan, S. *et al.* Problems in early diagnosis of bladder cancer in a spinal cord injury patient: report of a case of simultaneous production of granulocyte colony stimulating factor and parathyroid hormone-related protein by squamous cell carcinoma of urinary bladder. *BMC urology* **2**, 8 (2002).

28 Vaidyanathan, S., Soni, B. M., Bingley, J., Brown, E. & Markey, S. Prevention of pressure sore caused by indwelling urinary catheters. *Spinal cord* **40**, 489; author reply 490, doi:10.1038/sj.sc.3101356 (2002).

29 Kurosaka, Y. *et al.* A non-surgical rat model of foreign body-associated urinary tract infection with Pseudomonas aeruginosa. *Microbiology and immunology* **45**, 9-15 (2001).

30 Peychl, L. & Zalud, R. [Changes in the urinary bladder caused by short-term permanent catheter insertion]. *Casopis lekaru ceskych* **147**, 325-329 (2008).

31 Norden, C. W., Green, G. M. & Kass, E. H. Antibacterial mechanisms of the urinary bladder. *The Journal of clinical investigation* **47**, 2689-2700, doi:10.1172/JCI105952 (1968).

32 Parsons, C. L. Pathogenesis of urinary tract infections. Bacterial adherence, bladder defense mechanisms. *The Urologic clinics of North America* **13**, 563-568 (1986).

33 Saint, S. & Chenoweth, C. E. Biofilms and catheter-associated urinary tract infections. *Infectious disease clinics of North America* **17**, 411-432 (2003).

34 Warren, J. W. Catheter-associated urinary tract infections. *International journal of antimicrobial agents* **17**, 299-303 (2001).

35 Hashmi, S., Kelly, E., Rogers, S. O. & Gates, J. Urinary tract infection in surgical patients. *American journal of surgery* **186**, 53-56 (2003).

36 Garibaldi, R. A., Burke, J. P., Britt, M. R., Miller, M. A. & Smith, C. B. Meatal colonization and catheter-associated bacteriuria. *The New England journal of medicine* **303**, 316-318, doi:10.1056/NEJM198008073030605 (1980).

37 Stickler, D. J. Bacterial biofilms in patients with indwelling urinary catheters. *Nature clinical practice. Urology* **5**, 598-608, doi:10.1038/ncpuro1231 (2008).

38 Costerton, J. W. Introduction to biofilm. *International journal of antimicrobial agents* **11**, 217-221; discussion 237-219 (1999).

39 Costerton, J. W., Montanaro, L. & Arciola, C. R. Biofilm in implant infections: its production and regulation. *The International journal of artificial organs* **28**, 1062-1068 (2005).

40 Trautner, B. W. & Darouiche, R. O. Role of biofilm in catheter-associated urinary tract infection. *American journal of infection control* **32**, 177-183, doi:10.1016/j.ajic.2003.08.005 (2004).

41 Flemming, H. C., Neu, T. R. & Wozniak, D. J. The EPS matrix: the "house of biofilm cells". *Journal of bacteriology* **189**, 7945-7947, doi:10.1128/JB.00858-07 (2007).

42 Flemming, H. C. & Wingender, J. The biofilm matrix. *Nature reviews. Microbiology* **8**, 623-633, doi:10.1038/nrmicro2415 (2010).

43 Jacobsen, S. M., Stickler, D. J., Mobley, H. L. & Shirtliff, M. E. Complicated catheter-associated urinary tract infections due to Escherichia coli and Proteus mirabilis. *Clinical microbiology reviews* **21**, 26-59, doi:10.1128/CMR.00019-07 (2008).

44 O'Toole, G., Kaplan, H. B. & Kolter, R. Biofilm formation as microbial development. *Annual review of microbiology* **54**, 49-79, doi:10.1146/annurev.micro.54.1.49 (2000).

45 Roberts, A. P., Pratten, J., Wilson, M. & Mullany, P. Transfer of a conjugative transposon, Tn5397 in a model oral biofilm. *FEMS microbiology letters* **177**, 63-66 (1999).

46 Wang, B. Y., Chi, B. & Kuramitsu, H. K. Genetic exchange between Treponema denticola and Streptococcus gordonii in biofilms. *Oral microbiology and immunology* **17**, 108-112 (2002).

47 Branda, S. S., Vik, S., Friedman, L. & Kolter, R. Biofilms: the matrix revisited. *Trends in microbiology* **13**, 20-26, doi:10.1016/j.tim.2004.11.006 (2005).

48 Bridier, A., Briandet, R., Thomas, V. & Dubois-Brissonnet, F. Resistance of bacterial biofilms to disinfectants: a review. *Biofouling* **27**, 1017-1032, doi:10.1080/08927014.2011.626899 (2011).

49 Donlan, R. M. & Costerton, J. W. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical microbiology reviews* **15**, 167-193 (2002).

50 Jensen, E. T., Kharazmi, A., Lam, K., Costerton, J. W. & Hoiby, N. Human polymorphonuclear leukocyte response to Pseudomonas aeruginosa grown in biofilms. *Infect Immun* **58**, 2383-2385 (1990).

51 Jensen, P. O., Givskov, M., Bjarnsholt, T. & Moser, C. The immune system vs. Pseudomonas aeruginosa biofilms. *FEMS immunology and medical microbiology* **59**, 292-305, doi:10.1111/j.1574-695X.2010.00706.x (2010).

52 Lewis, K. Multidrug tolerance of biofilms and persister cells. *Current topics in microbiology and immunology* **322**, 107-131 (2008).

53 Yasuda, H., Ajiki, Y., Aoyama, J. & Yokota, T. Interaction between human polymorphonuclear leucocytes and bacteria released from in-vitro bacterial biofilm models. *Journal of medical microbiology* **41**, 359-367 (1994).

54 Lewis, K. Riddle of biofilm resistance. *Antimicrobial agents and chemotherapy* **45**, 999-1007, doi:10.1128/AAC.45.4.999-1007.2001 (2001).

55 Kucheria, R., Dasgupta, P., Sacks, S. H., Khan, M. S. & Sheerin, N. S. Urinary tract infections: new insights into a common problem. *Postgraduate medical journal* **81**, 83-86, doi:10.1136/pgmj.2004.023036 (2005).

56 Hidron, A. I. *et al.* NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America* **29**, 996-1011, doi:10.1086/591861 (2008).

57 Frank, D. N., Wilson, S. S., St Amand, A. L. & Pace, N. R. Culture-independent microbiological analysis of foley urinary catheter biofilms. *PloS one* **4**, e7811, doi:10.1371/journal.pone.0007811 (2009).

58 Macleod, S. M. & Stickler, D. J. Species interactions in mixed-community crystalline biofilms on urinary catheters. *Journal of medical microbiology* **56**, 1549-1557, doi:10.1099/jmm.0.47395-0 (2007).

59 Nielubowicz, G. R. & Mobley, H. L. Host-pathogen interactions in urinary tract infection. *Nature reviews. Urology* **7**, 430-441, doi:10.1038/nrurol.2010.101 (2010).

60 Mulvey, M. A. Adhesion and entry of uropathogenic Escherichia coli. *Cellular microbiology* **4**, 257-271 (2002).

61 Reid, G., van der Mei, H. C., Tieszer, C. & Busscher, H. J. Uropathogenic Escherichia coli adhere to urinary catheters without using fimbriae. *FEMS immunology and medical microbiology* **16**, 159-162 (1996).

62 Mulvey, M. A. *et al.* Induction and evasion of host defenses by type 1-piliated uropathogenic Escherichia coli. *Science* **282**, 1494-1497 (1998).

63 Fernandez, L. A. & Berenguer, J. Secretion and assembly of regular surface structures in Gram-negative bacteria. *FEMS microbiology reviews* **24**, 21-44 (2000).

64 Sauer, F. G., Mulvey, M. A., Schilling, J. D., Martinez, J. J. & Hultgren, S. J. Bacterial pili: molecular mechanisms of pathogenesis. *Current opinion in microbiology* **3**, 65-72 (2000).

65 Wu, X. R., Sun, T. T. & Medina, J. J. In vitro binding of type 1-fimbriated Escherichia coli to uroplakins Ia and Ib: relation to urinary tract infections. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 9630-9635 (1996).

66 Connell, I. *et al.* Type 1 fimbrial expression enhances Escherichia coli virulence for the urinary tract. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 9827-9832 (1996).

67 Hunstad, D. A. & Justice, S. S. Intracellular lifestyles and immune evasion strategies of uropathogenic Escherichia coli. *Annual review of microbiology* **64**, 203-221, doi:10.1146/annurev.micro.112408.134258 (2010).

68 Dhakal, B. K., Kulesus, R. R. & Mulvey, M. A. Mechanisms and consequences of bladder cell invasion by uropathogenic Escherichia coli. *European journal of clinical investigation* **38 Suppl 2**, 2-11, doi:10.1111/j.1365-2362.2008.01986.x (2008).

69 Bower, J. M., Eto, D. S. & Mulvey, M. A. Covert operations of uropathogenic Escherichia coli within the urinary tract. *Traffic* **6**, 18-31, doi:10.1111/j.1600-0854.2004.00251.x (2005).

70 Zhou, G. *et al.* Uroplakin Ia is the urothelial receptor for uropathogenic Escherichia coli: evidence from in vitro FimH binding. *Journal of cell science* **114**, 4095-4103 (2001).

71 Martinez, J. J., Mulvey, M. A., Schilling, J. D., Pinkner, J. S. & Hultgren, S. J. Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. *The EMBO journal* **19**, 2803-2812, doi:10.1093/emboj/19.12.2803 (2000).

72 Martinez, J. J. & Hultgren, S. J. Requirement of Rho-family GTPases in the invasion of Type 1-piliated uropathogenic Escherichia coli. *Cellular microbiology* **4**, 19-28 (2002).

73 Justice, S. S. *et al.* Differentiation and developmental pathways of uropathogenic Escherichia coli in urinary tract pathogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 1333-1338, doi:10.1073/pnas.0308125100 (2004).

74 Schwartz, D. J., Chen, S. L., Hultgren, S. J. & Seed, P. C. Population dynamics and niche distribution of uropathogenic Escherichia coli during acute and chronic urinary tract infection. *Infect Immun* **79**, 4250-4259, doi:10.1128/IAI.05339-11 (2011).

75 Wright, K. J., Seed, P. C. & Hultgren, S. J. Development of intracellular bacterial communities of uropathogenic Escherichia coli depends on type 1 pili. *Cellular microbiology* **9**, 2230-2241, doi:10.1111/j.1462-5822.2007.00952.x (2007).

76 Hannan, T. J., Mysorekar, I. U., Hung, C. S., Isaacson-Schmid, M. L. & Hultgren, S. J. Early severe inflammatory responses to uropathogenic E. coli predispose to chronic and recurrent urinary tract infection. *PLoS pathogens* **6**, e1001042, doi:10.1371/journal.ppat.1001042 (2010).

77 Mysorekar, I. U. & Hultgren, S. J. Mechanisms of uropathogenic Escherichia coli persistence and eradication from the urinary tract. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 14170-14175, doi:10.1073/pnas.0602136103 (2006).

78 Rosen, D. A., Hooton, T. M., Stamm, W. E., Humphrey, P. A. & Hultgren, S. J. Detection of intracellular bacterial communities in human urinary tract infection. *PLoS medicine* **4**, e329, doi:10.1371/journal.pmed.0040329 (2007).

79 Aberg, V. & Almqvist, F. Pilicides-small molecules targeting bacterial virulence. *Organic & biomolecular chemistry* **5**, 1827-1834, doi:10.1039/b702397a (2007).

80 Cegelski, L. *et al.* Small-molecule inhibitors target Escherichia coli amyloid biogenesis and biofilm formation. *Nature chemical biology* **5**, 913-919, doi:10.1038/nchembio.242 (2009).

81 Bouckaert, J. *et al.* Receptor binding studies disclose a novel class of high-affinity inhibitors of the Escherichia coli FimH adhesin. *Molecular microbiology* **55**, 441-455, doi:10.1111/j.1365-2958.2004.04415.x (2005).

82 Gouin, S. G., Wellens, A., Bouckaert, J. & Kovensky, J. Synthetic multimeric heptyl mannosides as potent antiadhesives of uropathogenic Escherichia coli. *ChemMedChem* **4**, 749-755, doi:10.1002/cmdc.200900034 (2009).

83 Pinkner, J. S. *et al.* Rationally designed small compounds inhibit pilus biogenesis in uropathogenic bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 17897-17902, doi:10.1073/pnas.0606795103 (2006).

84 Han, Z. *et al.* Structure-based drug design and optimization of mannoside bacterial FimH antagonists. *Journal of medicinal chemistry* **53**, 4779-4792, doi:10.1021/jm100438s (2010).

85 Schierholt, A., Hartmann, M. & Lindhorst, T. K. Bi- and trivalent glycopeptide mannopyranosides as inhibitors of type 1 fimbriae-mediated bacterial adhesion: variation of valency, aglycon and scaffolding. *Carbohydrate research* **346**, 1519-1526, doi:10.1016/j.carres.2011.04.023 (2011).

86 Schwardt, O. *et al.* Design, synthesis and biological evaluation of mannosyl triazoles as FimH antagonists. *Bioorganic & medicinal chemistry* **19**, 6454-6473, doi:10.1016/j.bmc.2011.08.057 (2011).

87 Cusumano, C. K. *et al.* Treatment and Prevention of Urinary Tract Infection with Orally Active FimH Inhibitors. *Science translational medicine* **3**, 109ra115, doi:10.1126/scitranslmed.3003021 (2011).

88 Fisher, K. & Phillips, C. The ecology, epidemiology and virulence of Enterococcus. *Microbiology* **155**, 1749-1757, doi:10.1099/mic.0.026385-0 (2009).

89 Giraffa, G. Enterococci from foods. *FEMS microbiology reviews* **26**, 163-171 (2002).

90 de Fatima Silva Lopes, M. *et al.* Antimicrobial resistance profiles of dairy and clinical isolates and type strains of enterococci. *International journal of food microbiology* **103**, 191-198, doi:10.1016/j.ijfoodmicro.2004.12.025 (2005).

91 Mohamed, J. A. & Huang, D. B. Biofilm formation by enterococci. *Journal of medical microbiology* **56**, 1581-1588, doi:10.1099/jmm.0.47331-0 (2007).

92 Mohamed, J. A., Huang, W., Nallapareddy, S. R., Teng, F. & Murray, B. E. Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by Enterococcus faecalis. *Infect Immun* **72**, 3658-3663, doi:10.1128/IAI.72.6.3658-3663.2004 (2004).

93 Joyanes, P., Pascual, A., Martinez-Martinez, L., Hevia, A. & Perea, E. J. In vitro adherence of Enterococcus faecalis and Enterococcus faecium to plastic biomaterials. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* **5**, 382-386 (1999).

94 Joyanes, P., Pascual, A., Martinez-Martinez, L., Hevia, A. & Perea, E. J. In vitro adherence of Enterococcus faecalis and Enterococcus faecium to urinary catheters. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* **19**, 124-127 (2000).

95 Sahm, D. F. *et al.* In vitro susceptibility studies of vancomycin-resistant Enterococcus faecalis. *Antimicrobial agents and chemotherapy* **33**, 1588-1591 (1989).

96 Ruggero, K. A., Schroeder, L. K., Schreckenberger, P. C., Mankin, A. S. & Quinn, J. P. Nosocomial superinfections due to linezolid-resistant Enterococcus faecalis: evidence for a gene dosage effect on linezolid MICs. *Diagnostic microbiology and infectious disease* **47**, 511-513 (2003).

97 Hidron, A. I., Schuetz, A. N., Nolte, F. S., Gould, C. V. & Osborn, M. K. Daptomycin resistance in Enterococcus faecalis prosthetic valve endocarditis. *The Journal of antimicrobial chemotherapy* **61**, 1394-1396, doi:10.1093/jac/dkn105 (2008).

98 Wiederhold, N. P., Coyle, E. A., Raad, II, Prince, R. A. & Lewis, R. E. Antibacterial activity of linezolid and vancomycin in an in vitro pharmacodynamic model of gram-positive catheter-related bacteraemia. *The Journal of antimicrobial chemotherapy* **55**, 792-795, doi:10.1093/jac/dki106 (2005).

99 Molinari, G., Pugliese, V., Schito, G. C. & Guzman, C. A. Bacteria involved in the blockage of biliary stents and their susceptibility to antibacterial agents. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* **15**, 88-92 (1996).

100 Uttley, A. H., Collins, C. H., Naidoo, J. & George, R. C. Vancomycin-resistant enterococci. *Lancet* **1**, 57-58 (1988).

101 Leclercq, R., Derlot, E., Duval, J. & Courvalin, P. Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium. *The New England journal of medicine* **319**, 157-161, doi:10.1056/NEJM198807213190307 (1988).

102 Rossolini, G. M., Mantengoli, E., Montagnani, F. & Pollini, S. Epidemiology and clinical relevance of microbial resistance determinants versus anti-Gram-positive agents. *Current opinion in microbiology* **13**, 582-588, doi:10.1016/j.mib.2010.08.006 (2010).

103 Thomas, V. C. *et al.* A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of Enterococcus faecalis. *Molecular microbiology* **72**, 1022-1036, doi:10.1111/j.1365-2958.2009.06703.x (2009).

104 Thomas, V. C., Thurlow, L. R., Boyle, D. & Hancock, L. E. Regulation of autolysis-dependent extracellular DNA release by Enterococcus faecalis extracellular proteases influences biofilm development. *Journal of bacteriology* **190**, 5690-5698, doi:10.1128/JB.00314-08 (2008).

105 Shankar, N. *et al.* Role of Enterococcus faecalis surface protein Esp in the pathogenesis of ascending urinary tract infection. *Infect Immun* **69**, 4366-4372, doi:10.1128/IAI.69.7.4366-4372.2001 (2001).

106 Kemp, K. D., Singh, K. V., Nallapareddy, S. R. & Murray, B. E. Relative contributions of Enterococcus faecalis OG1RF sortase-encoding genes, srtA and bps (srtC), to biofilm formation and a murine model of urinary tract infection. *Infect Immun* **75**, 5399-5404, doi:10.1128/IAI.00663-07 (2007).

107 Singh, K. V., Nallapareddy, S. R. & Murray, B. E. Importance of the ebp (endocarditis- and biofilm-associated pilus) locus in the pathogenesis of Enterococcus faecalis ascending urinary tract infection. *The Journal of infectious diseases* **195**, 1671-1677, doi:10.1086/517524 (2007).

108 Johnson, J. R., Clabots, C., Hirt, H., Waters, C. & Dunny, G. Enterococcal aggregation substance and binding substance are not major contributors to urinary tract colonization by Enterococcus faecalis in a mouse model of ascending unobstructed urinary tract infection. *Infect Immun* **72**, 2445-2448 (2004).

109 Kristich, C. J. *et al.* Development and use of an efficient system for random mariner transposon mutagenesis to identify novel genetic determinants of biofilm formation in the core Enterococcus faecalis genome. *Applied and environmental microbiology* **74**, 3377-3386, doi:10.1128/AEM.02665-07 (2008).

110 Costerton, J. W. & Stewart, P. S. Battling biofilms. *Scientific American* **285**, 74-81 (2001).

111 Stewart, P. S. & Costerton, J. W. Antibiotic resistance of bacteria in biofilms. *Lancet* **358**, 135-138 (2001).

112 Brady, R. A., Leid, J. G., Calhoun, J. H., Costerton, J. W. & Shirtliff, M. E. Osteomyelitis and the role of biofilms in chronic infection. *FEMS immunology and medical microbiology* **52**, 13-22, doi:10.1111/j.1574-695X.2007.00357.x (2008).

113 Costerton, J. W. Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. *Trends in microbiology* **9**, 50-52 (2001).

114 Ballering, K. S. *et al.* Functional genomics of Enterococcus faecalis: multiple novel genetic determinants for biofilm formation in the core genome. *Journal of bacteriology* **191**, 2806-2814, doi:10.1128/JB.01688-08 (2009).

115 Hancock, L. E. & Perego, M. The Enterococcus faecalis fsr two-component system controls biofilm development through production of gelatinase. *Journal of bacteriology* **186**, 5629-5639, doi:10.1128/JB.186.17.5629-5639.2004 (2004).

116 Hendrickx, A. P., Willems, R. J., Bonten, M. J. & van Schaik, W. LPxTG surface proteins of enterococci. *Trends in microbiology* **17**, 423-430, doi:10.1016/j.tim.2009.06.004 (2009).

117 Mohamed, J. A. & Murray, B. E. Influence of the fsr locus on biofilm formation by Enterococcus faecalis lacking gelE. *Journal of medical microbiology* **55**, 1747-1750, doi:10.1099/jmm.0.46729-0 (2006).

118 Pillai, S. K. *et al.* Effects of glucose on fsr-mediated biofilm formation in Enterococcus faecalis. *The Journal of infectious diseases* **190**, 967-970, doi:10.1086/423139 (2004).

119 Qin, X., Singh, K. V., Weinstock, G. M. & Murray, B. E. Effects of Enterococcus faecalis fsr genes on production of gelatinase and a serine protease and virulence. *Infect Immun* **68**, 2579-2586 (2000).

120 Qin, X., Singh, K. V., Weinstock, G. M. & Murray, B. E. Characterization of fsr, a regulator controlling expression of gelatinase and serine protease in Enterococcus faecalis OG1RF. *Journal of bacteriology* **183**, 3372-3382, doi:10.1128/JB.183.11.3372-3382.2001 (2001).

121 Thomas, V. C. & Hancock, L. E. Suicide and fratricide in bacterial biofilms. *The International journal of artificial organs* **32**, 537-544 (2009).

122 Barken, K. B. *et al.* Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in

Pseudomonas aeruginosa biofilms. *Environmental microbiology* **10**, 2331-2343, doi:10.1111/j.1462-2920.2008.01658.x (2008).

123 Kaplan, J. B., Jabbouri, S. & Sadovskaya, I. Extracellular DNA-dependent biofilm formation by Staphylococcus epidermidis RP62A in response to subminimal inhibitory concentrations of antibiotics. *Research in microbiology* **162**, 535-541, doi:10.1016/j.resmic.2011.03.008 (2011).

124 Kaplan, J. B. *et al.* Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in staphylococci. *The Journal of antibiotics*, doi:10.1038/ja.2011.113 (2011).

125 Martins, M. *et al.* Presence of extracellular DNA in the Candida albicans biofilm matrix and its contribution to biofilms. *Mycopathologia* **169**, 323-331, doi:10.1007/s11046-009-9264-y (2010).

126 Petersen, F. C., Pecharki, D. & Scheie, A. A. Biofilm mode of growth of Streptococcus intermedius favored by a competence-stimulating signaling peptide. *Journal of bacteriology* **186**, 6327-6331, doi:10.1128/JB.186.18.6327-6331.2004 (2004).

127 Qin, Z. *et al.* Role of autolysin-mediated DNA release in biofilm formation of Staphylococcus epidermidis. *Microbiology* **153**, 2083-2092, doi:10.1099/mic.0.2007/006031-0 (2007).

128 Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C. & Mattick, J. S. Extracellular DNA required for bacterial biofilm formation. *Science* **295**, 1487, doi:10.1126/science.295.5559.1487 (2002).

129 Rice, K. C. *et al.* The cidA murein hydrolase regulator contributes to DNA release and biofilm development in Staphylococcus aureus. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 8113-8118, doi:10.1073/pnas.0610226104 (2007).

130 Vollmer, W., Joris, B., Charlier, P. & Foster, S. Bacterial peptidoglycan (murein) hydrolases. *FEMS microbiology reviews* **32**, 259-286, doi:10.1111/j.1574-6976.2007.00099.x (2008).

131 Vollmer, W. & Bertsche, U. Murein (peptidoglycan) structure, architecture and biosynthesis in Escherichia coli. *Biochimica et biophysica acta* **1778**, 1714-1734, doi:10.1016/j.bbamem.2007.06.007 (2008).

132 Heilmann, C., Hussain, M., Peters, G. & Gotz, F. Evidence for autolysin-mediated primary attachment of Staphylococcus epidermidis to a polystyrene surface. *Molecular microbiology* **24**, 1013-1024 (1997).

133 Heilmann, C. *et al.* Identification and characterization of a novel autolysin (Aae) with adhesive properties from Staphylococcus epidermidis. *Microbiology* **149**, 2769-2778 (2003).

134 Eckert, C., Lecerf, M., Dubost, L., Arthur, M. & Mesnage, S. Functional analysis of AtlA, the major N-acetylglucosaminidase of Enterococcus faecalis. *Journal of bacteriology* **188**, 8513-8519, doi:10.1128/JB.01145-06 (2006).

135 Mesnage, S., Chau, F., Dubost, L. & Arthur, M. Role of N-acetylglucosaminidase and N-acetylmuramidase activities in Enterococcus faecalis peptidoglycan metabolism. *J Biol Chem* **283**, 19845-19853, doi:10.1074/jbc.M802323200 (2008).

136 Joris, B. *et al.* Modular design of the Enterococcus hirae muramidase-2 and Streptococcus faecalis autolysin. *FEMS microbiology letters* **70**, 257-264 (1992).

137 Beliveau, C., Potvin, C., Trudel, J., Asselin, A. & Bellemare, G. Cloning, sequencing, and expression in Escherichia coli of a Streptococcus faecalis autolysin. *Journal of bacteriology* **173**, 5619-5623 (1991).

138 Xu, Y., Jiang, L., Murray, B. E. & Weinstock, G. M. Enterococcus faecalis antigens in human infections. *Infect Immun* **65**, 4207-4215 (1997).

139 Qin, X., Singh, K. V., Xu, Y., Weinstock, G. M. & Murray, B. E. Effect of disruption of a gene encoding an autolysin of Enterococcus faecalis OG1RF. *Antimicrobial agents and chemotherapy* **42**, 2883-2888 (1998).

140 Bravetti, A. L. *et al.* Contribution of the autolysin AtlA to the bactericidal activity of amoxicillin against Enterococcus faecalis JH2-2. *Antimicrobial agents and chemotherapy* **53**, 1667-1669, doi:10.1128/AAC.00692-08 (2009).

141 Singh, K. V., Qin, X., Weinstock, G. M. & Murray, B. E. Generation and testing of mutants of Enterococcus faecalis in a mouse peritonitis model. *The Journal of infectious diseases* **178**, 1416-1420 (1998).

142 Spirig, T., Weiner, E. M. & Clubb, R. T. Sortase enzymes in Gram-positive bacteria. *Molecular microbiology* **82**, 1044-1059, doi:10.1111/j.1365-2958.2011.07887.x (2011).

143 Marraffini, L. A., Dedent, A. C. & Schneewind, O. Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria. *Microbiology and molecular biology reviews : MMBR* **70**, 192-221, doi:10.1128/MMBR.70.1.192-221.2006 (2006).

144 Kristich, C. J., Manias, D. A. & Dunny, G. M. Development of a method for markerless genetic exchange in Enterococcus faecalis and its use in construction of a srtA mutant. *Applied and environmental microbiology* **71**, 5837-5849, doi:10.1128/AEM.71.10.5837-5849.2005 (2005).

145 Bourgogne, A. *et al.* EbpR is important for biofilm formation by activating expression of the endocarditis and biofilm-associated pilus operon (ebpABC) of Enterococcus faecalis OG1RF. *Journal of bacteriology* **189**, 6490-6493, doi:10.1128/JB.00594-07 (2007).

146 Fabretti, F. *et al.* Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides. *Infect Immun* **74**, 4164-4171, doi:10.1128/IAI.00111-06 (2006).

147 Theilacker, C. *et al.* Glycolipids are involved in biofilm accumulation and prolonged bacteraemia in Enterococcus faecalis. *Molecular microbiology* **71**, 1055-1069, doi:10.1111/j.1365-2958.2009.06587.x (2009).

148 Hufnagel, M., Koch, S., Creti, R., Baldassarri, L. & Huebner, J. A putative sugar-binding transcriptional regulator in a novel gene locus in Enterococcus faecalis contributes to production of biofilm and prolonged bacteremia in mice. *The Journal of infectious diseases* **189**, 420-430, doi:10.1086/381150 (2004).

149 Kristich, C. J., Li, Y. H., Cvitkovitch, D. G. & Dunny, G. M. Esp-independent biofilm formation by Enterococcus faecalis. *Journal of bacteriology* **186**, 154-163 (2004).

150 Tendolkar, P. M., Baghdayan, A. S. & Shankar, N. Putative surface proteins encoded within a novel transferable locus confer a high-biofilm phenotype to

Enterococcus faecalis. *Journal of bacteriology* **188**, 2063-2072, doi:10.1128/JB.188.6.2063-2072.2006 (2006).

151 Spoering, A. L. & Gilmore, M. S. Quorum sensing and DNA release in bacterial biofilms. *Current opinion in microbiology* **9**, 133-137, doi:10.1016/j.mib.2006.02.004 (2006).

152 Steinberger, R. E. & Holden, P. A. Extracellular DNA in single- and multiple-species unsaturated biofilms. *Applied and environmental microbiology* **71**, 5404-5410, doi:10.1128/AEM.71.9.5404-5410.2005 (2005).

153 Vlassov, V. V., Laktionov, P. P. & Rykova, E. Y. Extracellular nucleic acids. *BioEssays : news and reviews in molecular, cellular and developmental biology* **29**, 654-667, doi:10.1002/bies.20604 (2007).

154 Bockelmann, U. *et al.* Bacterial extracellular DNA forming a defined network-like structure. *FEMS microbiology letters* **262**, 31-38, doi:10.1111/j.1574-6968.2006.00361.x (2006).

155 Bockelmann, U., Lunsdorf, H. & Szewzyk, U. Ultrastructural and electron energy-loss spectroscopic analysis of an extracellular filamentous matrix of an environmental bacterial isolate. *Environmental microbiology* **9**, 2137-2144, doi:10.1111/j.1462-2920.2007.01325.x (2007).

156 Jurcisek, J. A. *et al.* The PilA protein of non-typeable Haemophilus influenzae plays a role in biofilm formation, adherence to epithelial cells and colonization of the mammalian upper respiratory tract. *Molecular microbiology* **65**, 1288-1299, doi:10.1111/j.1365-2958.2007.05864.x (2007).

157 Hong, W., Juneau, R. A., Pang, B. & Swords, W. E. Survival of bacterial biofilms within neutrophil extracellular traps promotes nontypeable Haemophilus influenzae persistence in the chinchilla model for otitis media. *Journal of innate immunity* **1**, 215-224, doi:10.1159/000205937 (2009).

158 Cornett, J. B., Redman, B. E. & Shockman, G. D. Autolytic defective mutant of Streptococcus faecalis. *Journal of bacteriology* **133**, 631-640 (1978).

159 van Merode, A. E., van der Mei, H. C., Busscher, H. J. & Krom, B. P. Influence of culture heterogeneity in cell surface charge on adhesion and biofilm formation by Enterococcus faecalis. *Journal of bacteriology* **188**, 2421-2426, doi:10.1128/JB.188.7.2421-2426.2006 (2006).

160 van Merode, A. E., van der Mei, H. C., Busscher, H. J., Waar, K. & Krom, B. P. Enterococcus faecalis strains show culture heterogeneity in cell surface charge. *Microbiology* **152**, 807-814, doi:10.1099/mic.0.28460-0 (2006).

161 Levesque, C. M. *et al.* Involvement of sortase anchoring of cell wall proteins in biofilm formation by Streptococcus mutans. *Infect Immun* **73**, 3773-3777, doi:10.1128/IAI.73.6.3773-3777.2005 (2005).

162 Yamaguchi, M. *et al.* Role of Streptococcus sanguinis sortase A in bacterial colonization. *Microbes and infection / Institut Pasteur* **8**, 2791-2796, doi:10.1016/j.micinf.2006.08.010 (2006).

163 Suree, N., Jung, M. E. & Clubb, R. T. Recent advances towards new anti-infective agents that inhibit cell surface protein anchoring in Staphylococcus aureus and other gram-positive pathogens. *Mini reviews in medicinal chemistry* **7**, 991-1000 (2007).

164 Maresso, A. W. & Schneewind, O. Sortase as a target of anti-infective therapy. *Pharmacological reviews* **60**, 128-141, doi:10.1124/pr.107.07110 (2008).

165 Nallapareddy, S. R., Qin, X., Weinstock, G. M., Hook, M. & Murray, B. E. Enterococcus faecalis adhesin, ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. *Infect Immun* **68**, 5218-5224 (2000).

166 Nallapareddy, S. R. *et al.* Conservation of Ebp-type pilus genes among Enterococci and demonstration of their role in adherence of Enterococcus faecalis to human platelets. *Infect Immun* **79**, 2911-2920, doi:10.1128/IAI.00039-11 (2011).

167 Nallapareddy, S. R., Singh, K. V., Sillanpaa, J., Zhao, M. & Murray, B. E. Relative contributions of Ebp Pili and the collagen adhesin ace to host extracellular matrix protein adherence and experimental urinary tract infection by Enterococcus faecalis OG1RF. *Infect Immun* **79**, 2901-2910, doi:10.1128/IAI.00038-11 (2011).

168 Bourgogne, A. *et al.* Large scale variation in Enterococcus faecalis illustrated by the genome analysis of strain OG1RF. *Genome biology* **9**, R110, doi:10.1186/gb-2008-9-7-r110 (2008).

169 Creti, R. *et al.* Surface protein EF3314 contributes to virulence properties of Enterococcus faecalis. *The International journal of artificial organs* **32**, 611-620 (2009).

170 O'Gara, J. P. ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus. *FEMS microbiology letters* **270**, 179-188, doi:10.1111/j.1574-6968.2007.00688.x (2007).

171 Lewis, K. Programmed death in bacteria. *Microbiology and molecular biology reviews : MMBR* **64**, 503-514 (2000).

172 Mohamed, J. A. & Murray, B. E. Lack of correlation of gelatinase production and biofilm formation in a large collection of Enterococcus faecalis isolates. *Journal of clinical microbiology* **43**, 5405-5407, doi:10.1128/JCM.43.10.5405-5407.2005 (2005).

173 Pfeffer, J. M., Strating, H., Weadge, J. T. & Clarke, A. J. Peptidoglycan O acetylation and autolysin profile of Enterococcus faecalis in the viable but nonculturable state. *Journal of bacteriology* **188**, 902-908, doi:10.1128/JB.188.3.902-908.2006 (2006).

174 Lopez, D., Vlamakis, H. & Kolter, R. Biofilms. *Cold Spring Harbor perspectives in biology* **2**, a000398, doi:10.1101/cshperspect.a000398 (2010).

175 Chuang-Smith, O. N., Wells, C. L., Henry-Stanley, M. J. & Dunny, G. M. Acceleration of Enterococcus faecalis biofilm formation by aggregation substance expression in an ex vivo model of cardiac valve colonization. *PloS one* **5**, e15798, doi:10.1371/journal.pone.0015798 (2010).

176 Schlievert, P. M., Chuang-Smith, O. N., Peterson, M. L., Cook, L. C. & Dunny, G. M. Enterococcus faecalis endocarditis severity in rabbits is reduced by IgG Fabs interfering with aggregation substance. *PloS one* **5**, doi:10.1371/journal.pone.0013194 (2010).

177 Hartstein, A. I., Garber, S. B., Ward, T. T., Jones, S. R. & Morthland, V. H. Nosocomial urinary tract infection: a prospective evaluation of 108 catheterized patients. *Infection control : IC* **2**, 380-386 (1981).

178 Tambyah, P. A., Halvorson, K. T. & Maki, D. G. A prospective study of pathogenesis of catheter-associated urinary tract infections. *Mayo Clinic proceedings. Mayo Clinic* **74**, 131-136 (1999).

179 Norden, C. W. Study of urinary infections by catheterization. *The New England journal of medicine* **278**, 966-967 (1968).

180 Kaufman, J. M. *et al.* Bladder cancer and squamous metaplasia in spinal cord injury patients. *The Journal of urology* **118**, 967-971 (1977).

181 Jacobs, S. C. & Kaufman, J. M. Complications of permanent bladder catheter drainage in spinal cord injury patients. *The Journal of urology* **119**, 740-741 (1978).

182 Dedeic-Ljubovic, A. & Hukic, M. Catheter-related urinary tract infection in patients suffering from spinal cord injuries. *Bosnian journal of basic medical sciences / Udruzenje basicnih mediciniskih znanosti = Association of Basic Medical Sciences* **9**, 2-9 (2009).

183 Desai, P. J., Pandit, D., Mathur, M. & Gogate, A. Prevalence, identification and distribution of various species of enterococci isolated from clinical specimens with special reference to urinary tract infection in catheterized patients. *Indian journal of medical microbiology* **19**, 132-137 (2001).

184 Johnson, J. R., Dykstra, D., Brown, J. J., Kringstad, B. & Pryor, J. L. A surface swab method for culturing Foley catheters assays the pericatheter (urethral) but

not the urine (luminal) microbial population. *The Journal of laboratory and clinical medicine* **130**, 102-109 (1997).

185 Seno, Y., Kariyama, R., Mitsuhata, R., Monden, K. & Kumon, H. Clinical implications of biofilm formation by Enterococcus faecalis in the urinary tract. *Acta medica Okayama* **59**, 79-87 (2005).

186 Baldassarri, L. *et al.* Glycosaminoglycans mediate invasion and survival of Enterococcus faecalis into macrophages. *The Journal of infectious diseases* **191**, 1253-1262, doi:10.1086/428778 (2005).

187 Montgomerie, J. Z., Kalmanson, G. M. & Guze, L. B. Virulence of enterococci in experimental pyelonephritis. *Urological research* **5**, 99-102 (1977).

188 Hirose, T., Kumamoto, Y., Tanaka, N., Yoshioka, M. & Tsukamoto, T. Study on pathogenesis of Enterococcus faecalis in urinary tract. *Urological research* **17**, 125-129 (1989).

189 Tsuchimori, N., Hayashi, R., Shino, A., Yamazaki, T. & Okonogi, K. Enterococcus faecalis aggravates pyelonephritis caused by Pseudomonas aeruginosa in experimental ascending mixed urinary tract infection in mice. *Infect Immun* **62**, 4534-4541 (1994).

190 Kau, A. L. *et al.* Enterococcus faecalis tropism for the kidneys in the urinary tract of C57BL/6J mice. *Infect Immun* **73**, 2461-2468, doi:10.1128/IAI.73.4.2461-2468.2005 (2005).

191 Rosen, D. A., Hung, C. S., Kline, K. A. & Hultgren, S. J. Streptozocin-induced diabetic mouse model of urinary tract infection. *Infect Immun* **76**, 4290-4298, doi:10.1128/IAI.00255-08 (2008).

192 Creti, R. *et al.* Survey for virulence determinants among Enterococcus faecalis isolated from different sources. *Journal of medical microbiology* **53**, 13-20 (2004).

193 Jankoska, G., Trajkovska-Dokic, E., Panovski, N., Popovska-Jovanovska, K. & Petrovska, M. Virulence factors and antibiotic resistance in Enterococcus faecalis isolated from urine samples. *Prilozi / Makedonska akademija na naukite i umetnostite, Oddelenie za bioloski i medicinski nauki = Contributions / Macedonian Academy of Sciences and Arts, Section of Biological and Medical Sciences* **29**, 57-66 (2008).

194 Kadurugamuwa, J. L. *et al.* Noninvasive biophotonic imaging for monitoring of catheter-associated urinary tract infections and therapy in mice. *Infect Immun* **73**, 3878-3887, doi:10.1128/IAI.73.7.3878-3887.2005 (2005).

195 Hamill, T. M., Gilmore, B. F., Jones, D. S. & Gorman, S. P. Strategies for the development of the urinary catheter. *Expert review of medical devices* **4**, 215-225, doi:10.1586/17434440.4.2.215 (2007).

196 Saint, S., Lipsky, B. A. & Goold, S. D. Indwelling urinary catheters: a one-point restraint? *Annals of internal medicine* **137**, 125-127 (2002).

197 Geppetti, P., Nassini, R., Materazzi, S. & Benemei, S. The concept of neurogenic inflammation. *BJU international* **101 Suppl 3**, 2-6, doi:10.1111/j.1464-410X.2008.07493.x (2008).

198 Bjorling, D. E., Beckman, M. & Saban, R. Neurogenic inflammation of the bladder. *Advances in experimental medicine and biology* **539**, 551-583 (2003).

199 Snider, R. M. *et al.* A potent nonpeptide antagonist of the substance P (NK1) receptor. *Science* **251**, 435-437 (1991).

200 Snider, R. M., Longo, K. P., Drozda, S. E., Lowe, J. A., 3rd & Leeman, S. E. Effect of CP-96,345, a nonpeptide substance P receptor antagonist, on salivation in rats. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 10042-10044 (1991).

201 McLean, S. *et al.* Pharmacology of CP-99,994; a nonpeptide antagonist of the tachykinin neurokinin-1 receptor. *The Journal of pharmacology and experimental therapeutics* **267**, 472-479 (1993).

202 McLean, S. *et al.* CP-99,994, a nonpeptide antagonist of the tachykinin NK1 receptor. *Regulatory peptides* **46**, 329-331 (1993).

203 Abraham, P., Rabi, S. & Kulothungan, P. Aminoguanidine, selective nitric oxide synthase inhibitor, ameliorates cyclophosphamide-induced hemorrhagic cystitis by inhibiting protein nitration and PARS activation. *Urology* **73**, 1402-1406, doi:10.1016/j.urology.2008.10.039 (2009).

204 Abraham, P., Rabi, S. & Selvakumar, D. Protective effect of aminoguanidine against oxidative stress and bladder injury in cyclophosphamide-induced hemorrhagic cystitis in rat. *Cell biochemistry and function* **27**, 56-62, doi:10.1002/cbf.1534 (2009).

205 Griffiths, M. J., Messent, M., MacAllister, R. J. & Evans, T. W. Aminoguanidine selectively inhibits inducible nitric oxide synthase. *British journal of pharmacology* **110**, 963-968 (1993).

206 Trevisani, M. *et al.* The influence of alpha1-adrenoreceptors on neuropeptide release from primary sensory neurons of the lower urinary tract. *European urology* **52**, 901-908, doi:10.1016/j.eururo.2007.01.016 (2007).

207 Stroncek, D. F., Skubitz, K. M. & McCullough, J. J. Biochemical characterization of the neutrophil-specific antigen NB1. *Blood* **75**, 744-755 (1990).

208 Santin, M., Motta, A., Denyer, S. P. & Cannas, M. Effect of the urine conditioning film on ureteral stent encrustation and characterization of its protein composition. *Biomaterials* **20**, 1245-1251 (1999).

209 Ike, Y., Craig, R. A., White, B. A., Yagi, Y. & Clewell, D. B. Modification of Streptococcus faecalis sex pheromones after acquisition of plasmid DNA. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 5369-5373 (1983).

210 Guiton, P. S. *et al.* Contribution of autolysin and Sortase a during Enterococcus faecalis DNA-dependent biofilm development. *Infect Immun* **77**, 3626-3638, doi:10.1128/IAI.00219-09 (2009).

211 An, F. Y., Sulavik, M. C. & Clewell, D. B. Identification and characterization of a determinant (eep) on the Enterococcus faecalis chromosome that is involved in production of the peptide sex pheromone cAD1. *Journal of bacteriology* **181**, 5915-5921 (1999).

212 Chandler, J. R. & Dunny, G. M. Characterization of the sequence specificity determinants required for processing and control of sex pheromone by the intramembrane protease Eep and the plasmid-encoded protein PrgY. *Journal of bacteriology* **190**, 1172-1183, doi:10.1128/JB.01327-07 (2008).

213 Frank, K. L. *et al.* Use of Recombinase-based in vivo Expression Technology to Characterize Enterococcus faecalis Gene Expression During Infection Identifies in vivo-Expressed Antisense RNAs and Implicates the Protease Eep in Pathogenesis. *Infect Immun*, doi:10.1128/IAI.05964-11 (2011).

214 Bjorling, D. E., Wang, Z. Y. & Bushman, W. Models of inflammation of the lower urinary tract. *Neurourology and urodynamics* **30**, 673-682, doi:10.1002/nau.21078 (2011).

215 Nishii, H., Nomura, M., Fujimoto, N. & Matsumoto, T. Up-regulation of interleukin-6 gene expression in cyclophosphamide-induced cystitis in mice: An in situ hybridization histochemical study. *International journal of urology : official journal of the Japanese Urological Association* **13**, 1339-1343, doi:10.1111/j.1442-2042.2006.01536.x (2006).

216 Olivar, T. & Laird, J. M. Cyclophosphamide cystitis in mice: behavioural characterisation and correlation with bladder inflammation. *Eur J Pain* **3**, 141-149, doi:10.1053/eujp.1998.0105 (1999).

217 O'Mahony, D. S., Pham, U., Iyer, R., Hawn, T. R. & Liles, W. C. Differential constitutive and cytokine-modulated expression of human Toll-like receptors in primary neutrophils, monocytes, and macrophages. *International journal of medical sciences* **5**, 1-8 (2008).

218 Schroder, N. W. *et al.* Lipoteichoic acid (LTA) of Streptococcus pneumoniae and Staphylococcus aureus activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2

are not involved. *J Biol Chem* **278**, 15587-15594, doi:10.1074/jbc.M212829200 (2003).

219 Daley, J. M., Thomay, A. A., Connolly, M. D., Reichner, J. S. & Albina, J. E. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *Journal of leukocyte biology* **83**, 64-70, doi:10.1189/jlb.0407247 (2008).

220 Ronald, A. The etiology of urinary tract infection: traditional and emerging pathogens. *The American journal of medicine* **113 Suppl 1A**, 14S-19S (2002).

221 Thurlow, L. R., Thomas, V. C., Fleming, S. D. & Hancock, L. E. Enterococcus faecalis capsular polysaccharide serotypes C and D and their contributions to host innate immune evasion. *Infect Immun* **77**, 5551-5557, doi:10.1128/IAI.00576-09 (2009).

222 Arciola, C. R. *et al.* Strong biofilm production, antibiotic multi-resistance and high gelE expression in epidemic clones of Enterococcus faecalis from orthopaedic implant infections. *Biomaterials* **29**, 580-586, doi:10.1016/j.biomaterials.2007.10.008 (2008).

223 Sillanpaa, J. *et al.* Characterization of the ebp(fm) pilus-encoding operon of Enterococcus faecium and its role in biofilm formation and virulence in a murine model of urinary tract infection. *Virulence* **1**, 236-246 (2010).

224 Hendrickx, A. P. *et al.* SgrA, a nidogen-binding LPXTG surface adhesin implicated in biofilm formation, and EcbA, a collagen binding MSCRAMM, are two novel adhesins of hospital-acquired Enterococcus faecium. *Infect Immun* **77**, 5097-5106, doi:10.1128/IAI.00275-09 (2009).

225 Patti, J. M., Allen, B. L., McGavin, M. J. & Hook, M. MSCRAMM-mediated adherence of microorganisms to host tissues. *Annual review of microbiology* **48**, 585-617, doi:10.1146/annurev.mi.48.100194.003101 (1994).

226 Sillanpaa, J. *et al.* A family of fibrinogen-binding MSCRAMMs from Enterococcus faecalis. *Microbiology* **155**, 2390-2400, doi:10.1099/mic.0.027821-0 (2009).

227 Sillanpaa, J., Prakash, V. P., Nallapareddy, S. R. & Murray, B. E. Distribution of genes encoding MSCRAMMs and Pili in clinical and natural populations of Enterococcus faecium. *Journal of clinical microbiology* **47**, 896-901, doi:10.1128/JCM.02283-08 (2009).

228 Rich, R. L. *et al.* Ace is a collagen-binding MSCRAMM from Enterococcus faecalis. *J Biol Chem* **274**, 26939-26945 (1999).

229 Mohamed, J. A., Teng, F., Nallapareddy, S. R. & Murray, B. E. Pleiotrophic effects of 2 Enterococcus faecalis sagA-like genes, salA and salB, which encode proteins that are antigenic during human infection, on biofilm formation and binding to collagen type i and fibronectin. *The Journal of infectious diseases* **193**, 231-240, doi:10.1086/498871 (2006).

230 Singh, K. V., Nallapareddy, S. R., Sillanpaa, J. & Murray, B. E. Importance of the collagen adhesin ace in pathogenesis and protection against Enterococcus faecalis experimental endocarditis. *PLoS pathogens* **6**, e1000716, doi:10.1371/journal.ppat.1000716 (2010).

231 Breton, Y. L. *et al.* Isolation and characterization of bile salts-sensitive mutants of Enterococcus faecalis. *Current microbiology* **45**, 434-439, doi:10.1007/s00284-002-3714-3 (2002).

232 Barford, J. M., Hu, Y., Anson, K. & Coates, A. R. A biphasic response from bladder epithelial cells induced by catheter material and bacteria: an in vitro study of the pathophysiology of catheter related urinary tract infection. *The Journal of urology* **180**, 1522-1526, doi:10.1016/j.juro.2008.06.012 (2008).

233 Cassatella, M. A. Neutrophil-derived proteins: selling cytokines by the pound. *Advances in immunology* **73**, 369-509 (1999).

234 Denkers, E. Y., Del Rio, L. & Bennouna, S. Neutrophil production of IL-12 and other cytokines during microbial infection. *Chemical immunology and allergy* **83**, 95-114 (2003).

235 Barreda, D. R., Hanington, P. C. & Belosevic, M. Regulation of myeloid development and function by colony stimulating factors. *Developmental and comparative immunology* **28**, 509-554, doi:10.1016/j.dci.2003.09.010 (2004).

236 Kobayashi, Y. Neutrophil infiltration and chemokines. *Critical reviews in immunology* **26**, 307-316 (2006).

237 Romano, M. *et al.* Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity* **6**, 315-325 (1997).

238 Kopf, M. *et al.* Pleiotropic defects of IL-6-deficient mice including early hematopoiesis, T and B cell function, and acute phase responses. *Annals of the New York Academy of Sciences* **762**, 308-318 (1995).

239 Ingersoll, M. A., Kline, K. A., Nielsen, H. V. & Hultgren, S. J. G-CSF induction early in uropathogenic Escherichia coli infection of the urinary tract modulates host immunity. *Cellular microbiology* **10**, 2568-2578, doi:10.1111/j.1462-5822.2008.01230.x (2008).

240 Eglezos, A., Giuliani, S., Viti, G. & Maggi, C. A. Direct evidence that capsaicin-induced plasma protein extravasation is mediated through tachykinin NK1 receptors. *European journal of pharmacology* **209**, 277-279 (1991).

241 Saban, R. *et al.* Mast cells mediate substance P-induced bladder inflammation through an NK(1) receptor-independent mechanism. *American journal of physiology. Renal physiology* **283**, F616-629, doi:10.1152/ajprenal.00096.2002 (2002).

242 Saban, R., Saban, M. R., Nguyen, N. B., Hammond, T. G. & Wershil, B. K. Mast cell regulation of inflammation and gene expression during antigen-induced bladder inflammation in mice. *Physiological genomics* **7**, 35-43 (2001).

243 Chavez, A., Smith, M. & Mehta, D. New insights into the regulation of vascular permeability. *International review of cell and molecular biology* **290**, 205-248, doi:10.1016/B978-0-12-386037-8.00001-6 (2011).

244 Newton, R. Molecular mechanisms of glucocorticoid action: what is important? *Thorax* **55**, 603-613 (2000).

245 Lan, N. C. *et al.* Mechanisms of glucocorticoid hormone action. *Journal of steroid biochemistry* **20**, 77-88 (1984).

246 Norman, M. & Hearing, S. D. Glucocorticoid resistance - what is known? *Current opinion in pharmacology* **2**, 723-729 (2002).

247 Chung, K. F. *et al.* Difficult/therapy-resistant asthma: the need for an integrated approach to define clinical phenotypes, evaluate risk factors, understand pathophysiology and find novel therapies. ERS Task Force on Difficult/Therapy-Resistant Asthma. European Respiratory Society. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* **13**, 1198-1208 (1999).

248 Dalakas, M. C. Inflammatory myopathies: management of steroid resistance. *Current opinion in neurology* **24**, 457-462, doi:10.1097/WCO.0b013e32834a9589 (2011).

249 Park, S. J. & Shin, J. I. Complications of nephrotic syndrome. *Korean journal of pediatrics* **54**, 322-328, doi:10.3345/kjp.2011.54.8.322 (2011).

250 Ito, K., Chung, K. F. & Adcock, I. M. Update on glucocorticoid action and resistance. *The Journal of allergy and clinical immunology* **117**, 522-543, doi:10.1016/j.jaci.2006.01.032 (2006).

251 Lu, Y. S. *et al.* Effects of glucocorticoids on the growth and chemosensitivity of carcinoma cells are heterogeneous and require high concentration of functional glucocorticoid receptors. *World journal of gastroenterology : WJG* **11**, 6373-6380 (2005).

252 Strickland, I. *et al.* High constitutive glucocorticoid receptor beta in human neutrophils enables them to reduce their spontaneous rate of cell death in response to corticosteroids. *The Journal of experimental medicine* **193**, 585-593 (2001).

253 Goleva, E. *et al.* Increased glucocorticoid receptor beta alters steroid response in glucocorticoid-insensitive asthma. *American journal of respiratory and critical care medicine* **173**, 607-616, doi:10.1164/rccm.200507-1046OC (2006).

254 Leendertse, M. *et al.* The acute-phase response impairs host defence against Enterococcus faecium peritonitis. *Immunology* **128**, e335-342, doi:10.1111/j.1365-2567.2008.02967.x (2009).

255 Leendertse, M. *et al.* Neutrophils are essential for rapid clearance of Enterococcus faecium in mice. *Infect Immun* **77**, 485-491, doi:10.1128/IAI.00863-08 (2009).

256 Fazal, N. *et al.* Differential effector responses by circulating/blood and tissue/peritoneal neutrophils following burn combined with Enterococcus faecalis infection. *FEMS immunology and medical microbiology*, doi:10.1111/j.1574-695X.2011.00881.x (2011).

257 Tsuda, Y., Shigematsu, K., Kobayashi, M., Herndon, D. N. & Suzuki, F. Role of polymorphonuclear neutrophils on infectious complications stemming from Enterococcus faecalis oral infection in thermally injured mice. *J Immunol* **180**, 4133-4138 (2008).

258 Ma, Z. *et al.* Role of polymorphonuclear neutrophils in the clearance of Enterococcus faecalis derived from saliva and infected root canals. *Journal of endodontics* **37**, 346-352, doi:10.1016/j.joen.2010.11.033 (2011).

259 Leendertse, M. *et al.* TLR2-dependent MyD88 signaling contributes to early host defense in murine Enterococcus faecium peritonitis. *J Immunol* **180**, 4865-4874 (2008).

260 Rakita, R. M. *et al.* Enterococcus faecalis bearing aggregation substance is resistant to killing by human neutrophils despite phagocytosis and neutrophil activation. *Infect Immun* **67**, 6067-6075 (1999).

261 Vanek, N. N. *et al.* Enterococcus faecalis aggregation substance promotes opsonin-independent binding to human neutrophils via a complement receptor type 3-mediated mechanism. *FEMS immunology and medical microbiology* **26**, 49-60 (1999).

262 Teng, F., Jacques-Palaz, K. D., Weinstock, G. M. & Murray, B. E. Evidence that the enterococcal polysaccharide antigen gene (epa) cluster is widespread in Enterococcus faecalis and influences resistance to phagocytic killing of E. faecalis. *Infect Immun* **70**, 2010-2015 (2002).

263 Shon, W., Lim, S., Bae, K. S., Baek, S. & Lee, W. The expression of alpha4 integrins by human polymorphonuclear neutrophils in response to sonicated extracts of Enterococcus faecalis. *Journal of endodontics* **31**, 369-372 (2005).

264 Shigematsu, K., Asai, A., Kobayashi, M., Herndon, D. N. & Suzuki, F. Enterococcus faecalis translocation in mice with severe burn injury: a pathogenic role of CCL2 and alternatively activated macrophages (M2aMphi and M2cMphi). *Journal of leukocyte biology* **86**, 999-1005, doi:10.1189/jlb.0409235 (2009).

265 La Carbona, S. *et al.* Comparative study of the physiological roles of three peroxidases (NADH peroxidase, Alkyl hydroperoxide reductase and Thiol peroxidase) in oxidative stress response, survival inside macrophages and virulence of Enterococcus faecalis. *Molecular microbiology* **66**, 1148-1163, doi:10.1111/j.1365-2958.2007.05987.x (2007).

266 Verneuil, N. *et al.* Implication of (Mn)superoxide dismutase of Enterococcus faecalis in oxidative stress responses and survival inside macrophages. *Microbiology* **152**, 2579-2589, doi:10.1099/mic.0.28922-0 (2006).

267 Verneuil, N. *et al.* Effects of the Enterococcus faecalis hypR gene encoding a new transcriptional regulator on oxidative stress response and intracellular survival within macrophages. *Infect Immun* **72**, 4424-4431, doi:10.1128/IAI.72.8.4424-4431.2004 (2004).

268 Baldassarri, L. *et al.* Receptor-mediated endocytosis of biofilm-forming Enterococcus faecalis by rat peritoneal macrophages. *The Indian journal of medical research* **119 Suppl**, 131-135 (2004).

269 Baldassarri, L. *et al.* Enterococcus spp. produces slime and survives in rat peritoneal macrophages. *Med Microbiol Immunol* **190**, 113-120 (2001).

270 Sussmuth, S. D. *et al.* Aggregation substance promotes adherence, phagocytosis, and intracellular survival of Enterococcus faecalis within human macrophages and suppresses respiratory burst. *Infect Immun* **68**, 4900-4906 (2000).

271 Gentry-Weeks, C. R., Karkhoff-Schweizer, R., Pikis, A., Estay, M. & Keith, J. M. Survival of Enterococcus faecalis in mouse peritoneal macrophages. *Infect Immun* **67**, 2160-2165 (1999).

272 Kosmidis, C. I. & Chandrasekar, P. H. Management of gram-positive bacterial infections in patients with cancer. *Leuk Lymphoma*, doi:10.3109/10428194.2011.602770.

273 Wisplinghoff, H. *et al.* Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study.

Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **39**, 309-317, doi:10.1086/421946

CID32752 [pii] (2004).

274 Sastry, V. *et al.* Vancomycin-resistant enterococci: an emerging pathogen in immunosuppressed transplant recipients. *Transplant Proc* **27**, 954-955 (1995).

275 Bonten, M. J., Willems, R. & Weinstein, R. A. Vancomycin-resistant enterococci: why are they here, and where do they come from? *Lancet Infect Dis* **1**, 314-325, doi:S1473-3099(01)00145-1 [pii]

10.1016/S1473-3099(01)00145-1 (2001).

276 Warren, J. W., Muncie, H. L., Jr. & Hall-Craggs, M. Acute pyelonephritis associated with bacteriuria during long-term catheterization: a prospective clinicopathological study. *The Journal of infectious diseases* **158**, 1341-1346 (1988).

277 Donelli, G. & Guaglianone, E. Emerging role of Enterococcus spp in catheter-related infections: biofilm formation and novel mechanisms of antibiotic resistance. *J Vasc Access* **5**, 3-9 (2004).

278 Donlan, R. M. Biofilms and device-associated infections. *Emerging infectious diseases* **7**, 277-281 (2001).

279 Dickinson, G. M. & Bisno, A. L. Infections associated with indwelling devices: infections related to extravascular devices. *Antimicrobial agents and chemotherapy* **33**, 602-607 (1989).

280 Dickinson, G. M. & Bisno, A. L. Infections associated with indwelling devices: concepts of pathogenesis; infections associated with intravascular devices. *Antimicrobial agents and chemotherapy* **33**, 597-601 (1989).

281 Guiton, P. S., Hung, C. S., Hancock, L. E., Caparon, M. G. & Hultgren, S. J. Enterococcal biofilm formation and virulence in an optimized murine model of foreign body-associated urinary tract infections. *Infect Immun* **78**, 4166-4175, doi:10.1128/IAI.00711-10 (2010).

282 Mulvey, M. A., Schilling, J. D. & Hultgren, S. J. Establishment of a persistent Escherichia coli reservoir during the acute phase of a bladder infection. *Infect Immun* **69**, 4572-4579, doi:10.1128/IAI.69.7.4572-4579.2001 (2001).

283 McTaggart, L. A., Rigby, R. C. & Elliott, T. S. The pathogenesis of urinary tract infections associated with Escherichia coli, Staphylococcus saprophyticus and S. epidermidis. *Journal of medical microbiology* **32**, 135-141 (1990).

284 Schilling, J. D., Lorenz, R. G. & Hultgren, S. J. Effect of trimethoprim-sulfamethoxazole on recurrent bacteriuria and bacterial persistence in mice infected with uropathogenic Escherichia coli. *Infect Immun* **70**, 7042-7049 (2002).

285 Barnhart, M. M. & Chapman, M. R. Curli biogenesis and function. *Annual review of microbiology* **60**, 131-147, doi:10.1146/annurev.micro.60.080805.142106 (2006).

286 Ejernaes, K. Bacterial characteristics of importance for recurrent urinary tract infections caused by Escherichia coli. *Danish medical bulletin* **58**, B4187 (2011).

287 Martinez-Medina, M. *et al.* Biofilm formation as a novel phenotypic feature of adherent-invasive Escherichia coli (AIEC). *BMC microbiology* **9**, 202, doi:10.1186/1471-2180-9-202 (2009).

288 Naves, P. *et al.* Correlation between virulence factors and in vitro biofilm formation by Escherichia coli strains. *Microbial pathogenesis* **45**, 86-91, doi:10.1016/j.micpath.2008.03.003 (2008).

289 Rosen, D. A. *et al.* Molecular variations in Klebsiella pneumoniae and Escherichia coli FimH affect function and pathogenesis in the urinary tract. *Infect Immun* **76**, 3346-3356, doi:10.1128/IAI.00340-08 (2008).

290 Mobley, H. L., Chippendale, G. R., Tenney, J. H., Hull, R. A. & Warren, J. W. Expression of type 1 fimbriae may be required for persistence of Escherichia coli in the catheterized urinary tract. *Journal of clinical microbiology* **25**, 2253-2257 (1987).

291 Elliott, T. S., Reid, L., Rao, G. G., Rigby, R. C. & Woodhouse, K. Bladder irrigation or irritation? *British journal of urology* **64**, 391-394 (1989).

292 Wright, K. J., Seed, P. C. & Hultgren, S. J. Uropathogenic Escherichia coli flagella aid in efficient urinary tract colonization. *Infect Immun* **73**, 7657-7668, doi:10.1128/IAI.73.11.7657-7668.2005 (2005).

293 Prasad, A., Cevallos, M. E., Riosa, S., Darouiche, R. O. & Trautner, B. W. A bacterial interference strategy for prevention of UTI in persons practicing intermittent catheterization. *Spinal cord* **47**, 565-569, doi:10.1038/sc.2008.166 (2009).

294 Macovei, L. *et al.* Enterococcus faecalis with the gelatinase phenotype regulated by the fsr operon and with biofilm-forming capacity are common in the agricultural environment. *Environmental microbiology* **11**, 1540-1547, doi:10.1111/j.1462-2920.2009.01881.x (2009).

295 Prabhakara, R. *et al.* Suppression of the inflammatory immune response prevents the development of chronic biofilm infection due to methicillin-resistant Staphylococcus aureus. *Infect Immun* **79**, 5010-5018, doi:10.1128/IAI.05571-11 (2011).

296 Horvath, D. J., Jr. *et al.* Morphological plasticity promotes resistance to phagocyte killing of uropathogenic Escherichia coli. *Microbes and infection / Institut Pasteur* **13**, 426-437, doi:10.1016/j.micinf.2010.12.004 (2011).

297 Hunstad, D. A., Justice, S. S., Hung, C. S., Lauer, S. R. & Hultgren, S. J. Suppression of bladder epithelial cytokine responses by uropathogenic Escherichia coli. *Infect Immun* **73**, 3999-4006, doi:10.1128/IAI.73.7.3999-4006.2005 (2005).

298 Miyazaki, S. *et al.* Cytotoxic effect of hemolytic culture supernatant from Enterococcus faecalis on mouse polymorphonuclear neutrophils and macrophages. *Microbiology and immunology* **37**, 265-270 (1993).

299 Juneau, R. A., Pang, B., Weimer, K. E., Armbruster, C. E. & Swords, W. E. Nontypeable Haemophilus influenzae initiates formation of neutrophil extracellular traps. *Infect Immun* **79**, 431-438, doi:10.1128/IAI.00660-10 (2011).

300 Auer, J. & Seager, L. D. Experimental Local Bladder Edema Causing Urine Reflux into Ureter and Kidney. *The Journal of experimental medicine* **66**, 741-754 (1937).

301 Valle, J. *et al.* Broad-spectrum biofilm inhibition by a secreted bacterial polysaccharide. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 12558-12563, doi:10.1073/pnas.0605399103 (2006).

302 Blattner, F. R. *et al.* The complete genome sequence of Escherichia coli K-12. *Science* **277**, 1453-1462 (1997).

303 Chen, S. L. *et al.* Identification of genes subject to positive selection in uropathogenic strains of Escherichia coli: a comparative genomics approach. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 5977-5982, doi:10.1073/pnas.0600938103 (2006).

304 Harding, M. A. & Theodorescu, D. RhoGDI2: a new metastasis suppressor gene: discovery and clinical translation. *Urologic oncology* **25**, 401-406, doi:10.1016/j.urolonc.2007.05.006 (2007).

305 Chang, C. C. *et al.* Oct-3/4 expression reflects tumor progression and regulates motility of bladder cancer cells. *Cancer research* **68**, 6281-6291, doi:10.1158/0008-5472.CAN-08-0094 (2008).

306 Reis, L. O. *et al.* Evolution on experimental animal model for upper urothelium carcinogenesis. *World journal of urology* **28**, 499-505, doi:10.1007/s00345-010-0545-3 (2010).

307 Cohen, S. M., Ohnishi, T., Arnold, L. L. & Le, X. C. Arsenic-induced bladder cancer in an animal model. *Toxicology and applied pharmacology* **222**, 258-263, doi:10.1016/j.taap.2006.10.010 (2007).

308 Cohen, S. M., Ohnishi, T., Clark, N. M., He, J. & Arnold, L. L. Investigations of rodent urinary bladder carcinogens: collection, processing, and evaluation of urine and bladders. *Toxicologic pathology* **35**, 337-347, doi:10.1080/01926230701197115 (2007).

309 St John, A., Boyd, J. C., Lowes, A. J. & Price, C. P. The use of urinary dipstick tests to exclude urinary tract infection: a systematic review of the literature. *American journal of clinical pathology* **126**, 428-436, doi:10.1309/C69RW1BT7E4QAFPV (2006).

310 Trautner, B. W. *et al.* Increased expression of type-1 fimbriae by nonpathogenic Escherichia coli 83972 results in an increased capacity for catheter adherence and bacterial interference. *The Journal of infectious diseases* **198**, 899-906, doi:10.1086/591093 (2008).

311 Perez-Casal, J., Okada, N., Caparon, M. G. & Scott, J. R. Role of the conserved C-repeat region of the M protein of Streptococcus pyogenes. *Molecular microbiology* **15**, 907-916 (1995).

312 Perez-Casal, J. F., Dillon, H. F., Husmann, L. K., Graham, B. & Scott, J. R. Virulence of two Streptococcus pyogenes strains (types M1 and M3) associated with toxic-shock-like syndrome depends on an intact mry-like gene. *Infect Immun* **61**, 5426-5430 (1993).

313 Paulsen, I. T. *et al.* Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis. *Science* **299**, 2071-2074, doi:10.1126/science.1080613 (2003).

314 Kline, K. A. *et al.* Mechanism for sortase localization and the role of sortase localization in efficient pilus assembly in Enterococcus faecalis. *Journal of bacteriology* **191**, 3237-3247, doi:10.1128/JB.01837-08 (2009).

315 Granok, A. B., Parsonage, D., Ross, R. P. & Caparon, M. G. The RofA binding site in Streptococcus pyogenes is utilized in multiple transcriptional pathways. *Journal of bacteriology* **182**, 1529-1540 (2000).

316 Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 6640-6645, doi:10.1073/pnas.120163297 (2000).

317 Murphy, K. C. & Campellone, K. G. Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic E. coli. *BMC molecular biology* **4**, 11, doi:10.1186/1471-2199-4-11 (2003).

318 Chen, S. L. *et al.* Positive selection identifies an in vivo role for FimH during urinary tract infection in addition to mannose binding. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 22439-22444, doi:10.1073/pnas.0902179106 (2009).

319 Ewing, B. & Green, P. Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome research* **8**, 186-194 (1998).

320 Ewing, B., Hillier, L., Wendl, M. C. & Green, P. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome research* **8**, 175-185 (1998).

321 Thompson, J. D., Higgins, D. G. & Gibson, T. J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic acids research* **22**, 4673-4680 (1994).

322 Gilmore, K. S., Srinivas, P., Akins, D. R., Hatter, K. L. & Gilmore, M. S. Growth, development, and gene expression in a persistent Streptococcus gordonii biofilm. *Infect Immun* **71**, 4759-4766 (2003).

323 Ferrieres, L., Hancock, V. & Klemm, P. Specific selection for virulent urinary tract infectious Escherichia coli strains during catheter-associated biofilm formation. *FEMS immunology and medical microbiology* **51**, 212-219, doi:10.1111/j.1574-695X.2007.00296.x (2007).

324 Sizemore, R. K., Caldwell, J. J. & Kendrick, A. S. Alternate gram staining technique using a fluorescent lectin. *Applied and environmental microbiology* **56**, 2245-2247 (1990).

325 Schilling, J. D., Mulvey, M. A. & Hultgren, S. J. Dynamic interactions between host and pathogen during acute urinary tract infections. *Urology* **57**, 56-61 (2001).

326 Saria, A. & Lundberg, J. M. Evans blue fluorescence: quantitative and morphological evaluation of vascular permeability in animal tissues. *Journal of neuroscience methods* **8**, 41-49 (1983).

327 Beaudry, F. & Vachon, P. Determination of substance P in rat spinal cord by high-performance liquid chromatography electrospray quadrupole ion trap mass spectrometry. *Biomedical chromatography : BMC* **20**, 1344-1350, doi:10.1002/bmc.703 (2006).

328 Brown, R. W. & Chirala, R. Utility of microwave-citrate antigen retrieval in diagnostic immunohistochemistry. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **8**, 515-520 (1995).

329 Shi, S. R., Chaiwun, B., Young, L., Cote, R. J. & Taylor, C. R. Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* **41**, 1599-1604 (1993).

330 Justice, S. S., Lauer, S. R., Hultgren, S. J. & Hunstad, D. A. Maturation of intracellular Escherichia coli communities requires SurA. *Infect Immun* **74**, 4793-4800, doi:10.1128/IAI.00355-06 (2006).

331 Ike, Y., Clewell, D. B., Segarra, R. A. & Gilmore, M. S. Genetic analysis of the pAD1 hemolysin/bacteriocin determinant in Enterococcus faecalis: Tn917 insertional mutagenesis and cloning. *Journal of bacteriology* **172**, 155-163 (1990).

332 Wertman, K. F., Wyman, A. R. & Botstein, D. Host/vector interactions which affect the viability of recombinant phage lambda clones. *Gene* **49**, 253-262 (1986).

333 Hengstler, J. G., Fuchs, J. & Oesch, F. DNA strand breaks and DNA cross-links in peripheral mononuclear blood cells of ovarian cancer patients during chemotherapy with cyclophosphamide/carboplatin. *Cancer research* **52**, 5622-5626 (1992).

334 Reid, J. L. & Vincent, J. Clinical pharmacology and therapeutic role of prazosin and related alpha-adrenoceptor antagonists. *Cardiology* **73**, 164-174 (1986).